PHD

Studies on the antigenicity of citrate synthase

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Award date:
1989

Awarding institution:
University of Bath

STUDIES ON THE ANTIGENICITY OF CITRATE SYNTHASE

Submitted by

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for the degree of PhD

of the

University of Bath

1989

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to my supervisors, Dr. D. W. Hough and Dr. M. J. Danson, for their invaluable and inspiring guidance and suggestions given during the course of this study.

I wish to thank all my colleagues and the members of the Biochemistry department, who made my stay at Bath so enjoyable. I am grateful to Mrs A. Hudson for typing this manuscript. I would also like to thank my wife Suroor for her patience.

Finally, I would like to thank the Ministry of Higher Education and Scientific Research of the Republic of Iraq for the scholarship provided to me during the course of this study.

ABSTRACT

This Thesis describes a study of the antigenicity of the enzyme pig heart citrate synthase (PHCS).

A number of predictive methods based on the amino acid sequence (hydrophilicity and composite surface profile) and crystallographic structure (atomic mobility and protrusion) of PHCS were used to predict immunogenic peptides. These methods indicated that peptides at the region 288-302 (PCS-1) and the region 76-90 (PCS-2) should be important in the antigenic activity of PHCS. The selected peptides were synthesised by the Fmoc-solid phase method and used to produce anti-sera in rabbits.

Characterization of polyclonal and monoclonal antibodies originally raised against intact PHCS for their interaction with intact PHCS and the synthetic peptides, indicated that the polyclonal anti-serum contained antibodies reacting with native PHCS. The monoclonal antibody used showed reactivity with the enzyme in a denatured state. Monoclonal antibodies against PHCS were tested for their ability to bind to the synthetic peptide antigens PCS-1 and PCS-2. There was no evidence for specific binding of MAbs to either peptide in direct ELISA and soluble peptide did not inhibit the binding of MAb to intact PHCS. Furthermore, there was no evidence for specific binding of CNBr fragments of PHCS to an immunosorbent column containing immobilized MAb.

Anti-sera raised against the synthetic peptides showed a strict specificity for the immunizing peptide and both anti-peptide sera reacted with whole PHCS. However, a fraction of the antibody population in the anti-peptide sera was directed against denatured forms of the enzyme. Reactivity of anti-peptide sera with CNBr fragments of PHCS suggested that each antiserum reacts with the predicted fragment containing

the corresponding sequence of amino acids to the immunizing peptide.

A correlation was observed between the results of the experimental study of antigenicity of PHCS and the predictive methods. This observation confirms the validity of the use of a predictive approach to identify potential synthetic peptide immunogens from the amino acid sequence of an oligomeric protein.

TO

MY MOTHER

ABBREVIATIONS

A_{405}	Absorbance at 405 nm
B. megaterium	Bacillus megaterium
B. value	X-ray crystallographic temperature factor
CS	Citrate synthase
CoA	Coenzyme A
DMF	Dimethylformamide
DTNP	5,5'-dithiobis (2-nitrobenzoic acid)
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
FCA	Freund's complete adjuvant
Fmoc	9-Fluorenylmethoxycarbonyl
FPLC	Fast protein liquid chromatography
Influenza HA	Influenza haemagglutinin
MAb	Monoclonal antibody
Mb	Myoglobin
MOPS	3-(N-Morpholino) propanesulphonic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCS-1	Peptide one from PHCS
PCS-2	Peptide two from PHCS
PHCS	pig heart citrate synthase
SDS	Sodium dodecyl sulphate
swMb	Sperm whale myoglobin

TEMED	N,N,N′,N′-tetramethylethylenediamine
TFA	Trifluoroacetic acid
TMVP	Tobacco mosaic virus protein

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CHAPTER ONE IMMUNOGENICITY AND ANTIGENICITY OF PROTEIN

1.1 INTRODUCTION

The key reaction of an immune response is the binding of antigen to antibody, whether it be on the surface of a B lymphocyte, or in the body fluid itself. In considering the response of the immune system to a "foreign" protein, a clear distinction must be made between its immunogenic and antigenic characteristics. Immunogenicity refers to the ability to induce an immune response and to produce specific antibodies, while antigenicity refers to the ability to react with an antibody. From these definitions, it is apparent that an immunogenic compound will also behave as an antigen while the converse is not necessarily true. However, some low molecular weight substances, such as drugs, hormones and other small molecules, are unable to induce a primary response and are thus non-immunogenic unless conjugated to larger "carrier" molecules; such substances are termed "haptens".

1.2 PROTEIN ANTIGENIC STRUCTURE

Proteins are composed of a number of distinct antigenic regions, called antigenic determinants or epitopes, which are the sites of an antigen which bind an antibody. These antigenic determinants can be either continuous (residing in the linear sequence of residues), or discontinuous (made up of residues that are not contiguous in sequence, but are brought together at the protein surface by the folding of the polypeptide chain) (Benjamin et al., 1984; Atassi, 1978).

In this section, the discovery of different types of antigenic determinants will be described, using experiments employing serum antibodies.

Early workers used peptide fragments to study protein immunochemistry. As early as 1942, Landsteiner showed how peptides from silk fibroin were able to inhibit the reaction of the latter with its antibodies.

Work by Atassi and Collaborators from 1963-1975 (Atassi, 1975) led to the

elucidation of the first complete antigenic structure of a protein, that of sperm whale myoglobin (swMb). They also determined the antigenic sites of hen egg lysozyme (Atassi, 1978), serum albumin (Atassi, 1982) and human adult haemoglobin (Hb) (Atassi, 1984).

The antigenic structure of a protein cannot be determined by the exclusive application of a single approach. A strategy was, therefore, developed (Atassi, 1975) which relied on five approaches. These are:

1) The study of the effect of conformational changes on the immunochemical behaviour of the protein.

2) The study of the immunochemistry and conformation of specific chemically modified derivatives of the protein.

3) The isolation and characterization of immunochemically reactive fragments that can account quantitatively for the total reaction of the native protein.

4) The study of the immunochemistry and conformation of specific chemical derivatives of immunochemically reactive peptides.

5) The synthesis, chemically, of the reactive regions defined by method 1-4, and verification of the antigenic activity of these peptides.

Thus, determination of the antigenic structures of swMb and lysozyme has defined the problems involved in precise delineation of two types of antigen sites for subsequent workers studying protein antigenicity.

a) Main features of the antigenic structure of sperm whale myoglobin

Sperm whale myoglobin (swMb) is a protein of 153 amino acid residues, folded in a helical compact structure carrying 1 haem group (Edmundson, 1965).

Conformational changes in swMb caused by inclusion of modified haem groups, or modification of side chains, were shown to influence antigenic activity (Andres and Atassi 1970). A series of well purified derivatives, modified at a total of 23 amino

acid sites, gave an initial indication of the location of some of the amino acids needed for antibody binding. However, care was needed to ensure that a resultant decrease in antibody binding following modification was directly due to the alteration of an essential antigenic site residue and not caused indirectly by a conformational change (Atassi, 1975; Atassi and Thomas, 1969). Spectroscopic measurement suggested that modification of peptides did not result in a change of their conformation in solution (Atassi and Perlstein, 1973).

Peptide fragments of swMb and derivatives of the same were tested for their ability to react with antisera raised against the native swMb (Atassi, 1975; Singhal and Atassi, 1971). Following this initial description of the epitopes, synthetic peptides corresponding to these regions were tested for their ability to inhibit immunoprecipitation of native swMb by antisera (Pai and Atassi, 1975; Koketsu and Atassi, 1974).

By this method native swMb was shown to have 5 major antigenic sites, each consisting of a linear amino acid sequence. This type of antigenic site is termed "continuous" (also termed a linear epitope). Two of the sites exhibit degrees of shift or displacement and minor variability in size (limited to ±1 residue only) from one antiserum to the next. The sites contain 6-7 residues and are between 19-23 A in their extended dimensions (Atassi, 1984). The size, surface locations and shape of these antigenic sites make them quite accessible for binding with antibody combining sites. Due to their content of hydrophilic residues (lysine, arginine, aspartate, glutamate and histidine), it was concluded that the interaction with antibodies must be predominantly polar in nature. Other stabilizing effects are contributed by hydroxy and non-polar amino acids through hydrogen bonding and hydrophobic interactions (Atassi, 1984).

The same five antigenic sites of swMb are recognised by rabbit, goat, chicken, cat, pig and mouse antisera (Twining et al., 1980).

b) Main features of the antigenic structure of lysozyme

Hen egg lysozyme is structurally quite different from myoglobin. It has 129 amino acid residues and it is held together in a tight conformation by 4 internal disulphide bonds. Lysozyme has only 25% of its residues forming α-helices (Imoto et al., 1972), compared to 70% for myoglobin (Singhal and Atassi, 1970).

Initial work using the previously proposed strategy proved difficult, partly due to the tight conformation making specific cleavage to fragments unreliable and partly due to the apparent need for the disulphide bonds to be intact in order to retain antigenicity (Lee and Atassi, 1973; Habeeb and Atassi, 1971a). This initial work predicted 3 antigenic sites.

Attempts were made to chemically synthesize regions corresponding to these sites and containing the disulphide bonds, but this proved too difficult for routine use (Atassi et al. 1975). Diglycyl segments, however, were found to be ideal replacements for disulphides in chemical-synthesis experiments. This newly developed concept of chemically synthesising a region where the constituent amino acids are not generally found sequentially linked by peptide bonds in the native protein was called "surface-simulation synthesis" (Atassi et al. 1976a).

For antigenic site 2, the initially implicated residues from chemical modification experiments are shown in Fig. 1.1a (Atassi et al., 1976b; Lee and Atassi, 1975). Following synthesis of a variety of peptides of the type shown in Fig. 1.1b, the final constituent residues of the antigenic site, Fig. 1.1c, were delineated (Atassi, 1978; Lee and Atassi, 1976). This was the first antigenic determinant shown to be comprised of surface residues in close proximity by virtue of the folding of the polypeptide chain, but which are not sequentially linked by peptide bonds. This type of epitope is called a discontinuous antigenic determinant (sometimes termed topographic).

All three major antigenic site of lysozyme are found to be discontinuous, they each contain 5-6 residues and are between 21-30Å in their extended dimensions.

Fig. 1.1 Antigenic structure of Lysozyme

Fig. 1.1a

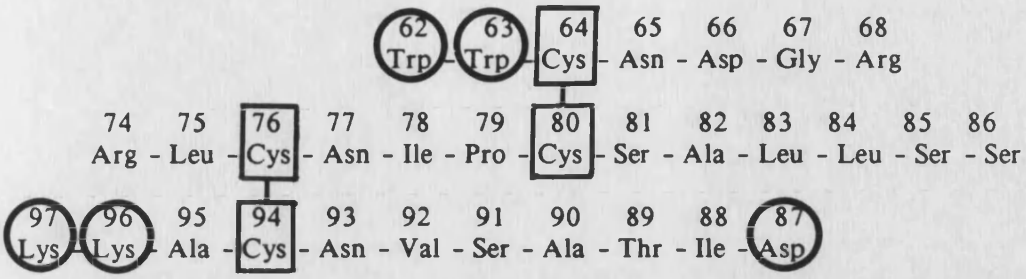


Fig. 1.1b Synthetic Site

Phe Gly Lys Lys Asn Thr Asp

<———— 2.16 nm ————>

Fig. 1.1c Constituent Residues

62 Trp — 97 Lys — 96 Lys — 93 Asn — 89 Thr — 87 Asp

0.71 0.41 0.56 0.51 0.54

<———— 2.73 ————>

1a) Shows the residues (ringed) thought to be important for the antigenicity of site 2 of hen egg lysozyme following fragmentation work.

1b) Shows the synthetic site found to carry the full reactivity of site 2.

1c) Shows the constitutent residues of the native site 2 of hen egg lysozyme. The distance (in nm) separating the constituent residues and the overall dimension of the site (in its extend form) are given, together with the dimension of the surface-simulation site (Atassi, 1978).

c) Level of response to regions outside the major antigenic sites

The number of antibodies that can be bound by the delineated synthetic sites of each of the above proteins (swMb and lysozyme) accounts for the bulk of the total antibodies raised against the respective protein in a given antiserum (approximately 98%). But, it should be noted that the regions outside the major antigenic sites are not entirely non-immunogenic, and that there is a very low level of antibody response (approximately 2%) versus these regions (Atassi, 1980; Atassi and Lee, 1978). Dean and Schechter (1979) found a subpopulation of less than 1% of total anti-Hb antibodies bound to an α129-141 epitope. This could only be detected when a large excess of antibody was used. Also, studies with synthetic reference non-antigenic regions of serum albumin revealed the presence of trace antibody binding accounting for 0.5-1.1% of the anti-protein antibodies in the antisera (Atassi, 1982; Sakata and Atassi, 1980).

d) Effects of amino acid substitutions and conformation on antigenic sites

The binding and recognition of a protein antigenic site was shown, during studies on the antigenic structure of myoglobin and lysozyme, to be highly dependent on the chemical characteristics of the residues constituting the site and on the conformational integrity of the site. Thus, alterations in the chemical nature of a side chain within a site (e.g. reversal or removal of a charge, creation of a new charge, elimination of a hydrogen bond) brought about either through chemical modification of a residue (Atassi et al., 1975, Atassi et al., 1973; Atassi and Thomas, 1969; Atassi, 1967) or its evolutionary replacement in a homologous protein, would cause a reduction or even complete elimination of the reactivity of the site (Atassi, 1978; Atassi, 1977; Atassi, 1975).

Conformational changes intentionally imposed on Mb and Hb by chemical alterations within the haem moiety (which resides outside the antigenic sites) lead in each protein to alterations in its antigenic reactivity (Atassi, 1967; Reichlin et al., 1963). Also, lysozyme and α-lactalbumin, two highly homologous proteins, do not exhibit any immunochemical cross-reaction, presumably because of conformation differences between the two proteins (Atassi and Habeeb, 1977; Habeeb and Atassi, 1971b). However, when the two proteins were unfolded by reduction and alkylation, they exhibited the expected immunochemical cross-reactions with antisera raised against either of the two unfolded proteins (Arnon and Maron, 1971).

Lee and Atassi (1977) showed that chemical modification (nitration) of a tyrosine outside an antigenic site of lysozyme creates an electrostatic inductive effect which disrupts the reactivity of that site. No detectable conformational changes were associated with this modification (Atassi, 1978).

Berzofsky et al. (1982) have concluded that 22% of myoglobin residues are involved in the antigenic sites, and yet 80% of evolutionary amino acid substitutions are immunologically detectable. Residues as far as 16Å distant from an antigenic site have been shown to exert conformational restrictions necessary for antibody binding at the site (Twining et al., 1980). If all the residues within 7Å of an antigenic site were arbitrarily considered to be important environmental residues, an additional 56% of Mb residues (giving a total of 78%) would be found to exert effects on its antigenicity (Kazim and Atassi, 1980).

Thus conformational changes in an antigen brought about by chemical modification or evolutionary change have been shown to be of great importance in influencing antigenicity. The extent and nature of conformational changes has a variable effect on antigenicity depending on the protein (Atassi et al., 1972; Andres and Atassi, 1970).

1.3 PREDICTION METHODS FOR LOCATING ANTIGENIC SITES IN PROTEINS

As mentioned above, proteins are composed of a number of distinct antigenic regions. These correspond to segments of the molecule which are accessible to an antibody and are specifically recognised by the antibody combining site. For example, antigenic sites for myoglobin, lysozyme, cytochrome C (Atassi, 1984; Benjamin et al., 1984) and influenza virus haemagglutinin (Green et al., 1982; Muller et al., 1982; Wiley et al., 1981) have been reported. Although the optimal epitope size to maximise antibody binding efficiency is generally accepted as being between 5-10 residues (Amit et al., 1985), some antibodies have been shown to be capable of binding to single amino acids and to dipeptides (Geysen, 1985). However, the precise nature of the interaction between a monoclonal antibody molecule and an epitope has been defined by X-ray crystallography of the immune complex with the enzyme lysozyme (Amit et al., 1986) and with influenza virus neurominidase (Colman et al., 1987).

Since the experimental analysis of antigenic sites is an arduous and time consuming procedure, much work is being performed to try and find a predictive approach for locating the antigenically important sites on a protein. Following correlation studies of known protein antigenic structures (found by experimental means) with various physical parameters, using computer analysis, some predictive methods have been suggested. These parameters include hydrophilicity, mobility, accessibility and surface protrusion. They are discussed in the following sections.

a) Hydrophilicity

Hydrophobic amino acids tend to be buried within the interior of globular proteins whereas hydrophilic ones tend to be on the exterior, where they can interact with

water in order for the protein to be stable in an aqueous environment (Van Regenmortel and de Marcillac, 1988). Kyte and Doolittle (1982) pointed that, ideally, the most satisfying way to determine the hydrophobic or hydrophilic inclinations of a given amino acid side-chain would be to measure its partition coefficient between water and a non-interacting, isotropic phase and to calculate from that partition coefficient a transfer free energy. So, they showed that, by evaluating the hydrophilic and hydrophobic character of stretches of residues along a polypeptide chain, it should be possible to distinguish regions of the sequence that are buried from those that are on the outside of the protein. This approach was used by Hopp and Woods (1981; 1983) and they suggested that the most hydrophilic segments of a sequence of various proteins would correlate well with antigenicity, because these segments are most likely to be on the external surface of a protein. Each amino acid was assigned a numerical value (hydrophilicity value) based on the polarity of its side chain. The hydrophilicity. values were based on solvent parameters which represent the measured free energy of transfer of the amino acid from water to ethanol (Levitt, 1976; Nozaki and Tanford, 1971). These values were repetitively averaged for overlapping sets of 6 residues along the peptide chain. Plotting these averages against the residue number of the leading residue gives a hydrophilicity profile, where a positive value indicates an above average hydrophilicity. Hoop and Woods (1981) in work carried out on 12 different proteins for which antigenic sites were known, showed that the point of highest local average hydrophilicity was invariably located in, or immediately adjacent, to an antigenic determinant.

This approach was then used to identify potential antigenic sites of hepatitis B surface antigen. Following chemical synthesis of the most hydrophilic peptide predicted, it was experimentally shown that this corresponded to a major antigenic determinant of that virus (Hopp and Woods, 1981; 1983). However, the secondary peaks of hydrophilicity do not always correlated well with known antigenic sites. For instance, Atassi (1984) has pointed out that only 2 of the 5 main antigenic sites of

myoglobin coincide with hydrophilicity maxima, and some hydrophilicity maxima are not antigenic. For influenza virus haemagglutinin, a strongly immunogenic region has been reported which is so hydrophobic that the corresponding synthetic peptide is insoluble in aqueous solvents (Atassi and Webster, 1983).

b) Accessibility

Atassi, 1984 showed that, for antibodies to bind a protein antigen in solution, antigenic sites should be accessible on the surface of the native protein. As defined, the accessibility of a group of atoms is proportional to its exposed surface area. So, the term "accessibility surface area" was introduced by Lee and Richards (1971) to describe the area over which contact between protein atoms and water molecules can occur. Molecular surface calculations can be performed on a protein of known structure by mathematically rolling a solvent water molecule over the van der Waal's surface of the protein (Lee and Richards, 1971). Novotny et al. (1986) have shown that antigenic determinants in proteins coincide with surface regions accessible to large probes comparable in size to an antibody combining region.

However, exceptions have been found to this requirement for antigenicity. When a MAb isolated from a mouse immunized with native swMb was used in a competitive ELISA, native swMb failed to compete for antibody with swMb bound directly to an ELISA plate. However, the MAb did bind swMb, horse Mb and a synthetic peptide by direct ELISA. Clearly this MAb recognises a sequential epitope which is only readily accessible when myoglobins are adsorbed onto a surface (Geysen, 1985). This might suggest that antigen (swMb) was denatured as a result of binding to the ELISA plate and that the MAb was directed against the denatured form.

Studies with monoclonal antibodies directed against the β_2-subunit of E. coli tryptophan synthase have shown that some antibodies bind rapidly to the native

antigen in solution, while others preferentially recognise the antigen only when it has been adsorbed onto the surface of ELISA plates (Friguet et al., 1984; Djavadi-Ohaniance et al., 1984).

Thus antibodies can be induced, during antigen presentation to epitopes which are inaccessible to antibody when the native antigen is in solution. However, this conclusion is based on characterization of the binding specificity of MAb. The relevance of these results to the overall antibody response, as represented by the antibody population is a polyclonal anti-serum, is unclear.

c) Protrusion index

This procedure is an extension of accessibility and takes account of the extent to which a residue protrudes out, from a globular protein, into the solvent. Thornton et al. (1986) calculated equimomental ellipsoids to fit proteins of known crystallographic structure, to evaluate the protrusion of a residue. A 90% ellipsoid included 90% of the atoms of the protein within it, with 10% lying outside or protruding from the globular shape. Each residue was assigned a "protrusion index" PI, specifying the ellipsoid at which that residue first becomes external. For instance, all residues outside the 90% ellipsoid were assigned a PI=9, those outside an 80% ellipsoid assigned PI=8, etc. Following assignment of PI values to all the amino acids of myoglobin, lysozyme and myohaemerythrin, plots were drawn of PI value versus residue number. A good correlation was found between residue PI maxima, and the locations of amino acids involved in known antigenic sites of these proteins.

For future use, where only the primary sequence is available, the same researchers have averaged the PI values of the amino acids found from known structures to give predicted protrusion values for each amino acid (Thornton et al., 1985). From the protein primary sequence, residue PI is plotted against amino acid sequence number in

order to identify likely protrusive regions.

d) Mobility

Protein molecules in solution are not static structures and their functional activity is often linked to dynamic conformational variations (Pain, 1983; Karplus and McCammon, 1983). Most of the information about local mobility (conformational changes) in proteins has been derived from nuclear magnetic resonance (NMR) studies and from the X-ray crystallographic refinement of protein structures (Ringe and Petsko, 1985). These refinement methods provide not only precise atomic coordinates, but also the atomic temperature factors, which are known as B factors (Frauenfelder et al., 1979). The temperature factor represents the mean-square displacement of each atom, and when plotted versus residue number provides a graphic image of the degree of mobility existing along the polypeptide chain (Williams and Moore, 1985).

Information from these techniques is not readily available at present for many proteins, and mobility predictions are certainly not applicable to cases where only the primary sequence is known (Berzofsky, 1985).

To study the relationship between antigenicity and mobility two different approaches have been used, the binding of antibodies to peptides and the binding of antibodies to proteins. Anti-protein antibodies are made against the most conformationally restricted states of the molecule, whereas anti-peptide antibodies are made against a conformationally less restricted epitope (Berzofsky, 1985).

Tainer et al. (1984) raised polyclonal antibodies against 12 synthetic peptides, each of between 10-15 amino acid residues, covering 70% of the exposed molecular surface area of the myohaemerythrin molecule, as defined by X-ray crystallography. A very good correlation was found between mobility of segments in the native protein and the ability of anti-peptide antibodies to bind to these sites on the native protein in

solution. This correlation was shown to be with the mobility of exposed surface regions, and not just due to surface exposure. However, highly exposed amino acids do tend to be less hydrophobic and due to their external relatively unconstrained position are more highly mobile than internal residues (Levitt, 1983).

Thus, the mobility of a segment of the native protein is a critical factor in determining the ability of antibodies against short peptides corresponding to that segment to bind to the native protein.

A second approach to examine the mobility of antigenic sites was to use antibodies against the native protein. The tobacco mosaic virus protein (TMVP) was shown to have 7 continuous antigenic determinants as identified using tryptic peptides (Altschuh et al., 1983; Milton and Van-Regenmortel, 1979). When the positions of these antigenic determinants were superimposed on a plot of temperature factors of main chain TMVP atoms, it was found that all seven antigenic regions corresponded to mobility peaks (Westhof et al., 1984). This correlation was better than that between antigenicity and either hydrophilicity or accessibility. Similarly, a good correlation was found between reported continuous antigenic sites of myoglobin and segmental mobility. These same investigators have concluded that the entire surface of the TMVP is antigenic, although composed primarily of discontinuous epitopes. Al Moudallal et al. (1985) suggested that no single criterion can be used to distinguish generally nonantigenic from antigenic regions.

One explanation for the apparent correlation found between antibody binding to continuous epitopes and mobility is an induced fit hypothesis (Marx, 1984; Tainer et al., 1984). In the induced fit model, an initial weak binding is stabilized by a conformational change in the antigen induced by the antibody.

Geysen (1985) has suggested how a thermodynamic analysis can be used to explain the interaction between antibody and antigen, allowing for the possibility of conformational changes occurring in both the antibody and the antigen. In contrast to these observations, Bahraoui et al. (1987) have reported that 2 peptides of 10 amino

acid residues in relatively rigid regions of the scorpion toxin are antigenic and capable of inducing the production of antibodies, which recognize the native toxin.

e) Composite surface profile

A combination of various parameters was investigated to improve the prediction of antigenic sites, since there is no single parameter able to predict all the antigenic sites on any protein. Recently a new set of amino acid hydrophilicity indices has been derived from the retention times of 20 model synthetic peptides in high-performance liquid chromatography (HPLC) (Parker et al., 1986). These researchers have chosen three parameters that individually provided the highest scores at predicting antigenic sites, to produce a composite predictive method. These parameters were HPLC hydrophilicity indices, predicted accessibility (Janin, 1979) and predicted flexibility B-values (Karplus and Schulz, 1985). The surface profile plots for the three parameters are combined by taking the maximum value for each residue in order to produce a composite surface profile. This composite profile showed good correlation with the known antigenic sites for several proteins, including myoglobin, lysozyme, cytochrome C and influenza HA1 (Parker et al., 1986). Van Regenmortel and de Marcillac (1988) have reported that this is the preferred method for antigenicity prediction.

f) Surface continuousness

The surface of globular protein, as defined by X-ray crystallographic coordinates, is very complex and convoluted. However, visual inspection of any structure shows that most risdues on the surface have neighbouring residues that are distant in the linear amino acid sequence (Barlow et al., 1986). As mentioned earlier in section 1.2,

protein antigenic determinants can be either continuous or discontinuous. Searches made for protein determinants using peptide fragments which compete with protein in antibody complex formation, or peptides that can be used to raise antibodies which crossreact with the native protein, are limited to the simulation of continuous determinants. However, different groups of workers (Benjamin et al., 1984; Van Regenmortel et al., 1984) have suggested that most determinants are discontinuous. Recently, Barlow et al. (1986) have used a computer method to search for "continuous patches" on the surface of a protein. The method involves centering a sphere of radius (r) on each surface atom in the protein, and calculating the proportion (F) of the other surface atoms enclosed by the sphere which belong to residues local in the amino acid sequence. If a sphere encloses only local surface atoms, that indicates a continuous patch. However, they have shown, by consideration of protein surfaces, that if the recognition zone between a protein and antibody has the same dimensions, about 20Å diameter, as those found for the lysozyme-antibody complex by Amit et al. (1986), none of the antibody-binding regions on a protein's surface will be "continuous". They have concluded also, that the parts of a protein's surface which are most continuous fall predominantly in the loops and/or protruding regions. This suggests that in order to predict the regions of a protein which provide the "best" antigenic peptides, methods such as: hydrophilicity; mobility; protrusion index, should be used since these are thought to identify loops and protruding regions.

g) Structurally inherent antigenic sites

Originally it was believed that the antigenic sites on a protein antigen comprise regions which differ in sequence from its counterpart in the immunized host (Urbanski and Margoliash, 1977).

Twining et al. (1980) found that the same five antigenic sites on swMb were

recognized by rabbit and goat antisera and by chicken, cat, pig and mouse antisera. Also, rabbit, goat, cat, and mouse antisera recognized the same three antigenic sites on lysozyme (Atassi, 1978; 1984). Atassi and his group concluded that antigenicity of the sites was independent of any sequence differences between the injected protein antigen and its counterpart in the immunized host (Atassi, 1984).

The antigenicity of proteins is conferred on certain surface regions by virtue of the three dimensional locations of these sites. Therefore it would follow that antigenic sites of conformationally related proteins are likely to have similar molecular locations. Using this concept it has been possible to predict by extrapolation of the three dimensional locations of the antigenic sites of swMb, many of the sites of human Hb (Kazim and Atassi, 1982) and also those of soybean leghaemoglobin, a distantly related haem protein (Hurrell et al., 1978).

Recently, a computer search has been performed to identify a set of peptide sequences, containing at least six amino acids, that occur in unrelated proteins (Wilson et al., 1985). Antibodies are to be raised against these peptides and tested for cross reactivity against them in their different conformations in the various proteins. In this way it will be possible to investigate further the roles of conformation and mobility in antibody binding.

1.4 STRUCTURAL DETERMINATION OF ANTIGEN-ANTIBODY COMPLEXES

The identification of antigenic regions on protein molecules has been attempted by a variety of methods, as mentioned above (section 1.3), but the structural basis of antigenicity remains unclear (Colman et al., 1987). However, Van Regenmortel (1987) pointed out that it should always be remembered that antigenicity is not a structural property per se but is a functional categorization that can be defined only by using antibody combining sites as a detecting device. Recently, an antigen binding fragment (Fab) of an antibody, together with its antigen, (Ag/Ab complex) has been crystallized. The three-dimensional structure of the complex between an antigen (lysozyme) and the Fab fragment from a monoclonal antibody against lysozyme has been determined and refined by X-ray crystallographic techniques (Amit et al., 1986). The structure of a complex between an antibody Fab fragment and influenza virus neuraminidase was analysed by the same techniques, as reported by Colman et al. (1987).

Amit et al. (1986) have explained that the interface involves 16 amino acid residues from the antigen and 17 residues from the antibody and extends over a large area, with maximum dimensions of about 30 x 20 Å. They concluded that the lysozyme antigenic determinants recognized by the monoclonal antibody are made up of two stretches of polypeptide chain, comprising residues 18-27 and 116-129, distant in the amino acid sequence but adjacent on the protein surface. This conclusion supports the observations made by Barlow et al. (1986), who argued that if the area on the surface of a protein that is recognized by antibody molecules is of the order of 20 x 25 Å, all protein epitopes are likely to be discontinuous. This argument rests on the premise that in all protein epitopes, the interaction occurs over a surface of about 20Å diameter. On the other hand, Van Regenmortel (1987) has reported that some residues in a continuous epitope can be replaced by any of the 19 amino acids without impairing the antigenic activity, whereas others cannot be substituted at all. This means that not every residue in the so-called continuous epitope is in contact with the

antibody and that the linear peptide is in fact antigenically discontinuous. It has been suggested by Westhof et al. (1984) that the antigenic regions of proteins are located in the more flexible chain segments, which may adopt a configuration that allows antibody binding. But, Amit et al. (1986) concluded in the case of their monoclonal Fab-lysozyme complex, that the parts of the lysozyme that make contact with antibody are not the more flexible regions of free lysozyme, and no deformation of the antigen nor any structural change in the Fab was observed. They found that antibody and antigen fit as "lock and key" with no detectable perturbation of the lysozyme structure.

Colman and his group (1987) found, in the case of an influenza virus neuraminidase-MAb complex, a structure which may be described as a square box (100 x 100 x 60 $\mathring{A}$) with four antennae (Fab molecules) attached to one surface on the outer corners. The size of the contact region formed with the Fab fragments of antibodies is about 700$\mathring{A}^2$ (unpublished data), and there are no more contacting residues than the 16 or 17 which were observed in the Fab-lysozyme complex by Amit et al. (1986). However, they concluded that the structure of the complex between a monoclonal antibody and neuraminidase showed features inconsistent with a rigid "lock and key" model for antibody-antigen interactions. They observed, in contrast to the findings from a lysozyme-antibody complex by (Amit et al., 1986), an unusual V_L-V_H pairing in the V module of the Fab, and local perturbation of the antigen at the centre of the epitope. Thus, the same group proposed that, during antigen binding, the V_L and V_H domains of the antibody slide at their interface, and that the nature of the V_L-V_H association may produce static or dynamic properties important for antigen binding. This suggests that there is a conformational change in the antigen and antibody as a result of binding. Therefore the interaction has some of the character of a "hand shake".

The two structural analyses to date of Fab-antigen complexes have provided rather different views of the way in which an antibody can bind to the surface of a protein. Sutton (1988) concluded, that these differences reflect the contrasting natures of the

two epitopes, and show that while mobility may sometimes be important in determining antigenicity, it is clearly not a necessary feature.

SUMMARY

Experimentally, to identify protein antigenic determinants, the methods available are:

1) Fragmentation of the native protein either by chemical cleavage or controlled proteolysis, followed by screening of the fragments for immunologically active components that bind to antibodies and can interfere with their interaction with the intact antigen.

2) Synthesis of peptides corresponding to potential epitopes, and measuring their reactivity with antibodies raised against the native protein.

and 3) Study of the antigen-monoclonal antibody (MAb) complex by X-ray crystallography.

By correlating the results obtained from experimental work with the predictive data (as discussed above) it should be possible to define the positions of the major antigenic sites on proteins. Most correlation studies reported in the literature have concentrated on small, monomeric proteins such as myoglobin, lysozyme, myohaemrythrin and tobacco mosaic virus protein. It is recognized that these are not necessarily representative of large, oligomeric proteins and that additional data are required before predictive methods can be reliably applied to more complex structures.

So, in this study I used the enzyme citrate synthase as an example of an oligomeric protein antigen because of the large amount of available structural and functional information on this enzyme.

CHAPTER TWO CITRATE SYNTHASE

2.1 THE CITRIC ACID CYCLE

The citric acid cycle operates in most living organisms representing a broad spectrum of diverse life styles. Such diversity may well be reflected in the roles that the citric acid cycle plays in order to suit the metabolic needs of a particular organism, be it provision of energy or of biochemical intermediates. These roles, in turn, could possibly be reflected in finer detail in terms of the structural, catalytic and regulatory properties of the enzymic machinery of the cycle. The citric acid cycle as shown in Fig. 2.1 was first proposed by Krebs and Johnson (1937) to describe the final stages of carbohydrate oxidation by animal tissues. It is now known that this metabolic cycle is at the core of cellular metabolism and serves as the final oxidation process of all major food-stuffs in all respiring organisms (Krebs and Lowenstein, 1960).

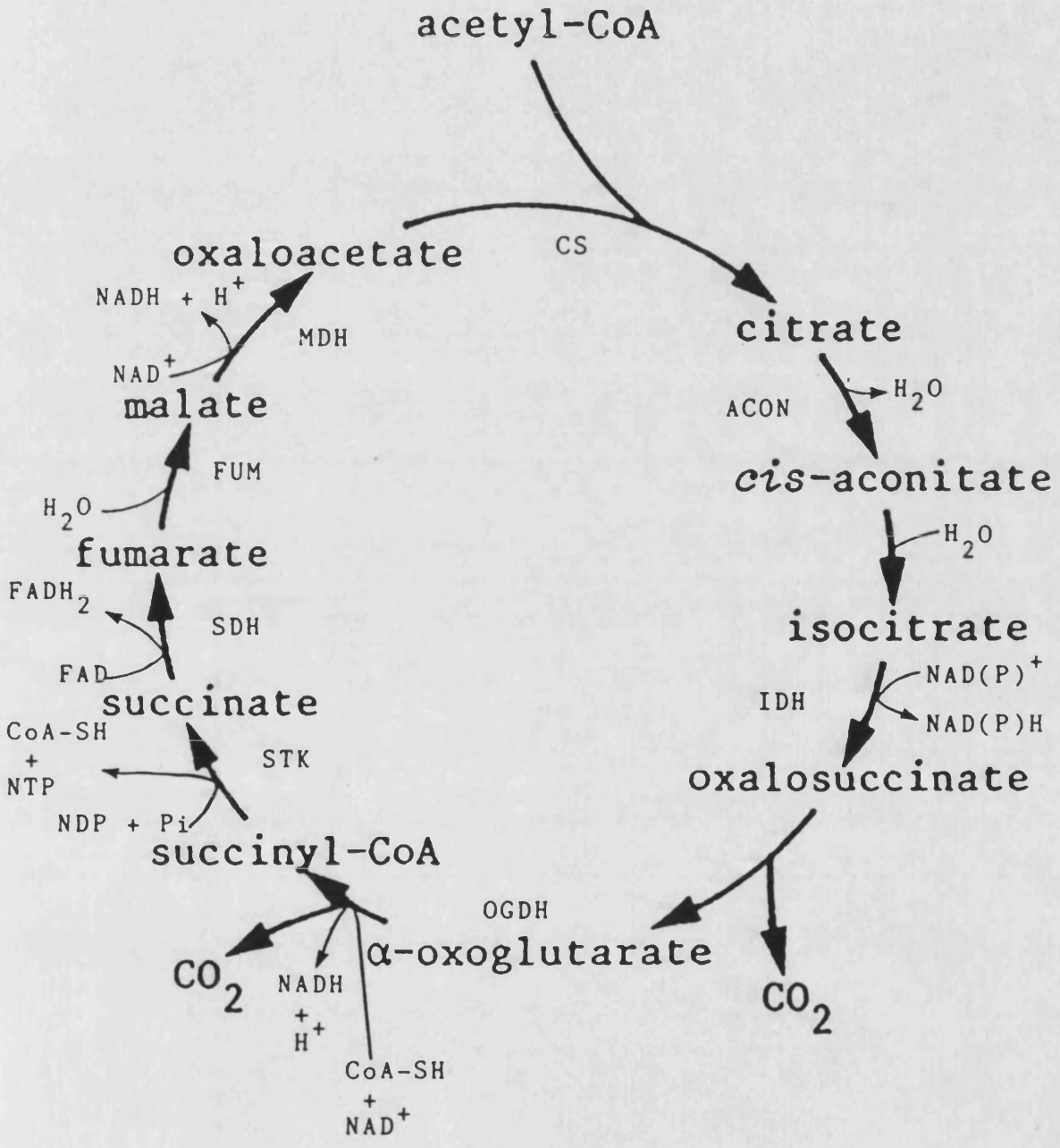
2.2 DISCOVERY OF CITRATE SYNTHASE

The "condensing enzyme" which brought about the combination of "active" acetate with oxaloacetate to form citrate, was first described by Stern et al. (1950). Later, "active" acetate was shown to be acetyl-CoA (Lynen and Reichert, 1951; Lynen et al., 1951) and it was thus established that "condensing enzyme", now known as citrate synthase (citrate oxaloacetate-Lyase (CoA acetylating); EC 4.1.3.7) catalyses the following reaction:

$$\text{Acetyl-S-CoA} + \text{oxaloacetate} + H_2O \longrightarrow \text{citrate} + \text{CoA-SH}$$

$$CH_3CO\text{-S-CoA} + HOOC\text{-}CO\text{-}CH_2\text{-}COOH + H_2O \longrightarrow HOOC\text{-}CH_2\text{-}\underset{\displaystyle OH}{\overset{\displaystyle COOH}{\overset{|}{\underset{|}{C}}}}\text{-}CH_2\text{-}COOH + \text{CoA-SH}$$

Fig. 2.1 The citric acid cycle



Citrate synthase has been extensively studied as it catalyses the first reaction of the citric acid cycle and has been shown to play an important regulatory role in a cell's metabolism (Danson, 1988; Beeckmans and Kanarek, 1984; Weitzman, 1981; Weitzman and Danson, 1976; Randle et al., 1970).

In eukaryotes the citric acid cycle enzymes are exclusively mitochondrial, with the exception of germinating plant seeds where citrate synthase, together with enzymes of the glyoxylate shunt, are also found in glyoxysomes (Tolbert, 1981). Citrate synthase is loosely attached to the inner mitochondrial membrane of eukaryotes (Dsouza and Srere, 1983) and is thought to be associated with the cell membrane of bacteria (Mitchell, 1963). Citrate synthase apparently has a close association with other enzymes such as pyruvate dehydrogenase (Sumegi et al., 1980), malate dehydrogenase and fumarase (Beckmans and Kanarek, 1981) and aspartate amino-transferase (Fahien and Kmiotek, 1983). Barnes and Weitzman (1986) have shown by gentle disruption of cells followed by gel filtration and sucrose density gradient centrifugation, that five sequential enzymes of the citric acid cycle (malate dehydrogenase, citrate synthase, aconitase, isocitrate dehydrogenase and fumarase) are specifically associated into a cluster.

2.3 DIVERSITY OF CITRATE SYNTHASES

Comparative studies of the citrate synthases of eukaryotes and eubacteria have shown that these enzymes possess a marked diversity of subunit structure, catalytic activity and allosteric regulation that shows a strong correlation with the taxonamic status of the source organism (Danson, 1988; Weitzman, 1981; Weitzman and Danson, 1976). A summary of these relationships is shown in Table 2.1.

Studies on citrate synthases of eukaryotic, and Gram positive bacteria showed that these enzymes are inhibited by ATP (Jangaard et al., 1968; Hathaway and Atkinson, 1965). However, Harford and Weitzman (1975) have reported that this inhibition is

Table 2.1 Cross-species properties of citrate synthase

	EUBACTERIA			ARCHAEBACTERIA			EUKARYOTES
	Gram negative						
	obligate aerobes	facultative anaerobes	Gram positive	Halio-philes	Thermo-acido-philes	Methan-ogens	
Mol. Wt.	270,000	270,000	90,000	110,000	85,000	-	90,000
Type	large	large	small	small	small	-	small
Subunit composition	hexamer	hexamer	dimer	-	dimer	-	dimer
INHIBITION							
NADH	allosteric (reactivated by AMP)	allosteric (AMP has no effect)	none	none	none	yes	v. little (isosteric)
2-oxo-glutarate	none	allosteric	none	none	none	yes	none
ATP	none	none	isosteric	weak	yes	yes	isosteric

isosteric, ATP competing with the substrate acetyl-CoA at the active site.

By contrast, the citrate synthases of Gram negative bacteria, are insensitive to ATP (Weitzman, 1966), but they are inhibited allosterically by NADH. Within this group, the enzymes of obligate aerobes can be reactivated by AMP, while those of facultative anaerobes cannot (Weitzman, 1981). Citrate synthases of Gram positive bacteria and eukaryotes, however, are generally much less inhibited by NADH, than those of Gram negative bacteria (Weitzman and Danson, 1976).

Inhibition of citrate synthase by 2-oxoglutarate was shown to be allosteric and only occurred in facultatively anaerobic Gram negative bacteria (Weitzman and Dunmore, 1969).

An investigation of the molecular sizes of the citrate synthases of Gram negative bacteria has shown these enzymes are hexameric molecules with molecular weight of approximately 270,000 (Robinson et al., 1983a; Tong and Duckworth, 1975). The citrate synthases of Gram positive bacteria and eukaryotic organisms have molecular weight values of approximately 90,000 and are dimers (Robinson et al., 1983b; Weitzman, 1981). However, it should be noted that the subunit sizes in both types of the enzyme, "large" and "small", are identical and are in the range 43,000-49,000.

As pointed out above, Gram positive bacterial citrate synthases are "small". There also exists another group of bacteria known as the Archaebacteria comprising the thermoacidophiles, halophiles and methanogens. These are not Gram positive, but they possess "small" citrate synthases. They represent a category of organisms which constitute a third evolutionary line of descent (Woese, 1981). In studies on thermoacidophile citrate synthases, they have been shown to be dimers of total molecular weight 85,000. They are sensitive to inhibition by ATP (Danson et al., 1985). Halophiles also have the small type of citrate synthase and are inhibited only weakly by ATP. Citrate synthase from the methanogen Methanosarcina barkeri was unusually inhibited by ATP, NADH and 2-oxoglutarate; however the enzyme is present in very low amounts and therefore its molecular size has not yet been

determined (Danson et al., 1985).

In summary, only the "large" citrate synthases have shown allosteric regulation, while the regulation of the "small" enzyme is isosteric.

A complementary approach has been used by Danson et al. (1979) to study the structure/function relationships of citrate synthases. This was achieved using the facultative anaerobe Escherichia coli. The "wild type" of this organism produces a heaxameric citrate synthase, which is sensitive to allosteric inhibition by both NADH and 2-oxoglutarate (Weitzman and Danson, 1976). From mutagenesis work, a mutant of E. coli was produced, in which the citrate synthase was now dimeric and was insensitive to inhibition by either NADH or 2-oxoglutarate (Danson et al., 1979). The apparently minor genetic alterations involved in this conversion emphasize the strong relationship between the structure and function of citrate synthase, and may mimic natural mutations which have occurred to produce the existing diversity of citrate synthases.

2.4 THE PRIMARY SEQUENCES OF CITRATE SYNTHASE FROM PIG HEART, YEAST, E. COLI R. PROWAZEKII AND A. ANITRATUM

Amino acid sequencing of fragments of pig heart citrate synthase (PHCS) produced by proteolytic (Bloxham et al., 1980) and chemical means, has provided the complete amino acid sequence of this enzyme (Bloxham et al., 1982; Bloxham et al., 1981), see Fig. 2.2.

For citrate synthase of E. coli, the primary sequence data were first obtained from sequencing the gene (Ner et al., 1983). Amino acid sequencing performed on fragments produced by proteolytic and chemical digests of E. coli citrate synthase has since confirmed the majority of this sequence (Bhayana and Duckworth, 1984).

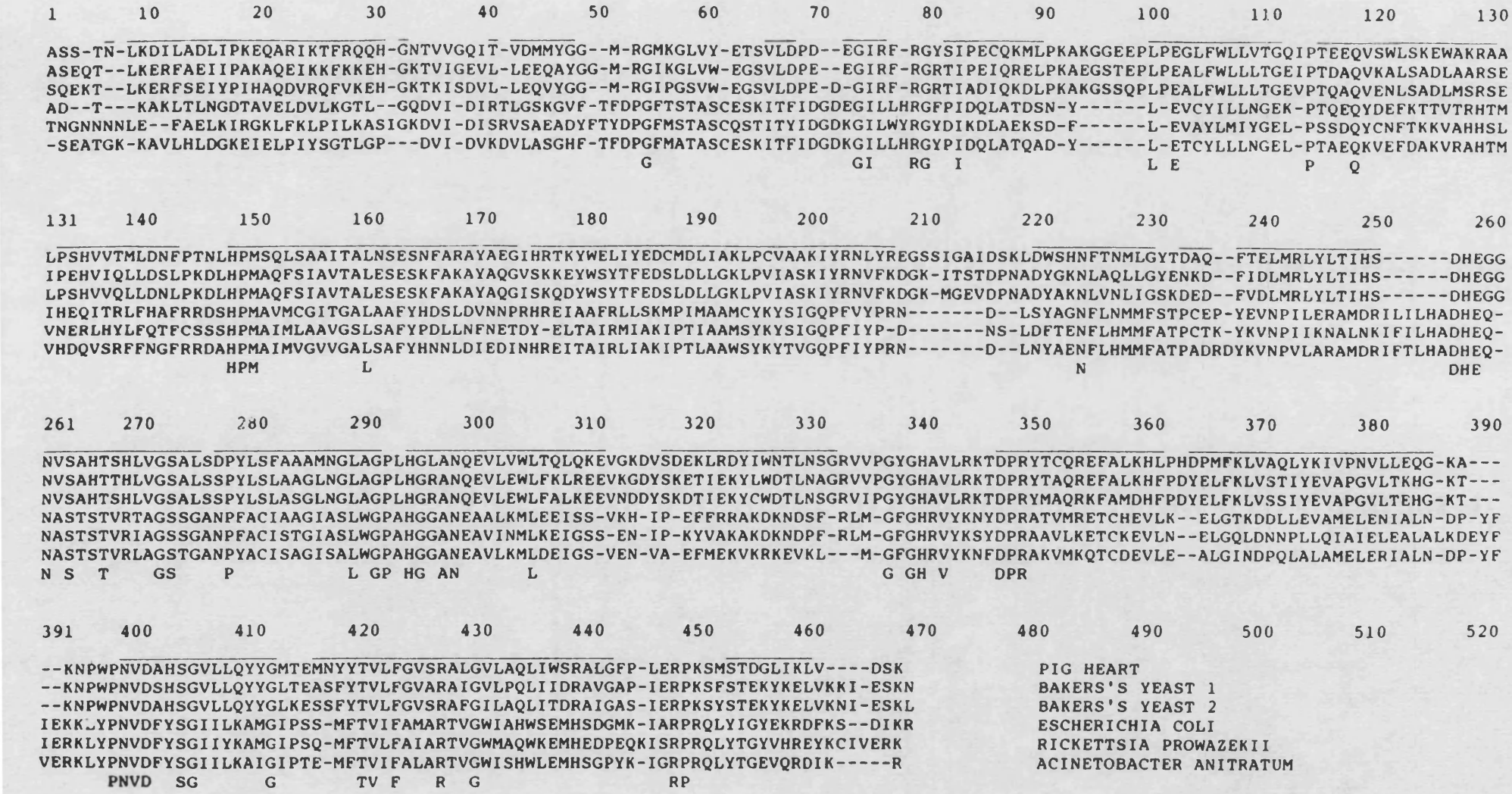
Suissa et al. (1984) have determined the nucleotide sequence of citrate synthase of

Fig. 2.2 Comparison between the amino acid sequences of citrate synthase from pig, heart, yeast 1,2, *E. coli*, *R. Prowazekii* and *A. anitratum*.

The sequences are aligned to maximise homologies.

Numbering relates to the sequence alignment and does not correspond to any single sequence.

This alignment supplied by Dr. C. Henneke.


1 10 20 30 40 50 60 70 80 90 100 110 120 130
ASS-TN-LKDILADLIPKEQARIKTFRQQH-GNTVVGQIT-VDMMYGG--M-RGMKGLVY-ETSVLDPD--EGIRF-RGYSIPECQKMLPKAKGGEEPLPEGLFWLLVTGQIPTEEQVSWLSKEWAKRAA
ASEQT--LKERFAEIIPAKAQEIKKFKKEH-GKTVIGEVL-LEEQAYGG-M-RGIKGLVW-EGSVLDPE--EGIRF-RGRTIPEIQRELPKAEGSTEPLPEALFWLLLTGEIPTDAQVKALSADLAARSE
SQEKT--LKERFSEIYPIHAQDVRQFVKEH-GKTKISDVL-LEQVYGG--M-RGIPGSVW-EGSVLDPE-D-GIRF-RGRTIADIQKDLPKAKGSSQPLPEALFWLLLTGEVPTQAQVENLSADLMSRSE
AD--T---KAKLTLNGDTAVELDVLKGTL--GQDVI-DIRTLGSKGVF-TFDPGFTSTASCESKITFIDGDEGILLHRGFPIDQLATDSN-Y------L-EVCYILLNGEK-PTQEQYDEFKTTVTRHTM
TNGNNNNLE--FAELKIRGKLFKLPILKASIGKDVI-DISRVSAEADYFTYDPGFMSTASCQSTITYIDGDKGILWYRGYDIKDLAEKSD-F------L-EVAYLMIYGEL-PSSDQYCNFTKKVAHHSL
-SEATGK-KAVLHLDGKEIELPIYSGTLGP---DVI-DVKDVLASGHF-TFDPGFMATASCESKITFIDGDKGILLHRGYPIDQLATQAD-Y------L-ETCYLLLNGEL-PTAEQKVEFDAKVRAHTM
131 140 150 160 170 180 190 200 210 220 230 240 250 260
LPSHVVTMLDNFPTNLHPMSQLSAAITALNSESNFARAYAEGIHRTKYWELIYEDCMDLIAKLPCVAAKIYRNLYREGSSIGAIDSKLDWSHNFTNMLGYTDAQ--FTELMRLYLTIHS------DHEGG
IPEHVIQLLDSLPKDLHPMAQFSIAVTALESESKFAKAYAQGVSKKEYWSYTFEDSLDLLGKLPVIASKIYRNVFKDGK-ITSTDPNADYGKNLAQLLGYENKD--FIDLMRLYLTIHS------DHEGG
LPSHVVQLLDNLPKDLHPMAQFSIAVTALESESKFAKAYAQGISKQDYWSYTFEDSLDLLGKLPVIASKIYRNVFKDGK-MGEVDPNADYAKNLVNLIGSKDED--FVDLMRLYLTIHS------DHEGG
IHEQITRLFHAFRRDSHPMAVMCGITGALAAFYHDSLDVNNPRHREIAAFRLLLSKMPIMAAMCYKYSIGQPFVYPRN-------D--LSYAGNFLNMMFSTPCEP-YEVNPILERAMDRILILHADHEQ-
VNERLHYLFQTFCSSSHPMAIMLAAVGSLSAFYPDLLNFNETDY-ELTAIRMIAKIPTIAAMSYKYSIGQPFIYP-D-------NS-LDFTENFLHMMFATPCTK-YKVNPIIKNALNKIFILHADHEQ-
VHDQVSRFFNGFRRDAHPMAIMVGVVGALSAFYHNNLDIEDINHREITAIRLIAKIPTLAAWSYKYTVGQPFIYPRN-------D--LNYAENFLHMMFATPADRDYKVNPVLARAMDRIFTLHADHEQ-
261 270 280 290 300 310 320 330 340 350 360 370 380 390
NVSAHTSHLVGSALSDPYLSFAAAMNGLAGPLHGLANQEVLVWLTQLQKEVGKDVSDEKLRDYIWNTLNSGRVVPGYGHAVLRKTDPRYTCQREFALKHLPHDPMFKLVAQLYKIVPNVLLEQG-KA---
NVSAHTTHLVGSALSSPYLSLAAGLNGLAGPLHGRANQEVLEWLFKLREEVKGDYSKETIEKYLWDTLNAGRVVPGYGHAVLRKTDPRYTAQREFALKHFPDYELFKLVSTIYEVAPGVLTKHG-KT---
NVSAHTSHLVGSALSSPYLSLASGLNGLAGPLHGRANQEVLEWLFALKEEVNDDYSKDTIEKYCWDTLNSGRVIPGYGHAVLRKTDPRYMAQRKFAMDHFPDYELFKLVSSIYEVAPGVLTEHG-KT---
NASTSTVRTAGSSGANPFACIAAGIASLWGPAHGGANEAALKMLEEISS-VKH-IP-EFFRRAKDKNDSF-RLM-GFGHRVYKNYDPRATVMRETCHEVLK--ELGTKDDLLEVAMELENIALN-DP-YF
NASTSTVRIAGSSGANPFACISTGIASLWGPAHGGANEAVINMLKEIGSS-EN-IP-KYVAKAKDKNDPF-RLM-GFGHRVYKSYDPRAAVLKETCKEVLN--ELGQLDNNPLLQIAIELEALALKDEYF
NASTSTVRLAGSTGANPYACISAGISALWGPAHGGANEAVLKMLDEIGS-VEN-VA-EFMEKVKRKEVKL---M-GFGHRVYKNFDPRAKVMKQTCDEVLE--ALGINDPQLALAMELERIALN-DP-YF
391 400 410 420 430 440 450 460 470 480 490 500 510 520
--KNPWPNVDAHSGVLLQYYGMTEMNYYTVLFGVSRALGVLAQLIWSRALGFP-LERPKSMSTDGLIKLV----DSK PIG HEART
--KNPWPNVDSHSGVLLQYYGLTEASFYTVLFGVARAIGVLPQLIIDRAVGAP-IERPKSFSTEKYKELVKKI-ESKN BAKERS'S YEAST 1
--KNPWPNVDAHSGVLLQYYGLKESSFYTVLFGVSRAFGILAQLITDRAIGAS-IERPKSYSTEKYKELVKNI-ESKL BAKERS'S YEAST 2
IEKKLYPNVDFYSGIILKAMGIPSS-MFTVIFAMARTVGWIAHWSEMHSDGMK-IARPRQLYIGYEKRDFKS--DIKR ESCHERICHIA COLI
IERKLYPNVDFYSGIIYKAMGIPSQ-MFTVLFAIARTVGWMAQWKEMHEDPEQKISRPRQLYTGYVHREYKCIVERK RICKETTSIA PROWAZEKII
VERKLYPNVDFYSGIILKAIGIPTE-MFTVIFALARTVGWISHWLEMHSGPYK-IGRPRQLYTGEVQRDIK-----R ACINETOBACTER ANITRATUM

yeast. The amino acid sequence, derived from the nucleotide sequence, shows that this citrate synthase has a strongly basic amino-terminal region (Fig. 2.2). It has been proposed that this amino-terminal region is removed during translocation of the precursor into the mitochondria.

Recently, DNA-derived sequences of Rickettsia Prowazekii (Wood et al., 1987) and Acinetobacter Anitratum (Donald and Duckworth, 1987) citrate cynthases have been reported, see Fig. 2.2.

It can be seen from Fig. 2.2, that a substantial degree of sequence resemblance exists between the citrate synthases of pig heart and yeast (Garforth, 1985). However, E. Coli citrate synthase shows limited regions of homology with both the citrate synthases from pig heart and yeast, but this is less than the homology between the two eukaryotic enzymes. Residues shown to be necessary for catalysis in PHCS are highly conserved in both the yeast and E. coli enzymes. These include His 235, His 238, His 320, Arg 329 and Asp 375 (Suissa et al., 1984).

Recently the sequence of the gene for CS from pig tissue has been determined (Evans et al., 1988). This sequence confirms the previous data obtained by amino acid sequencing.

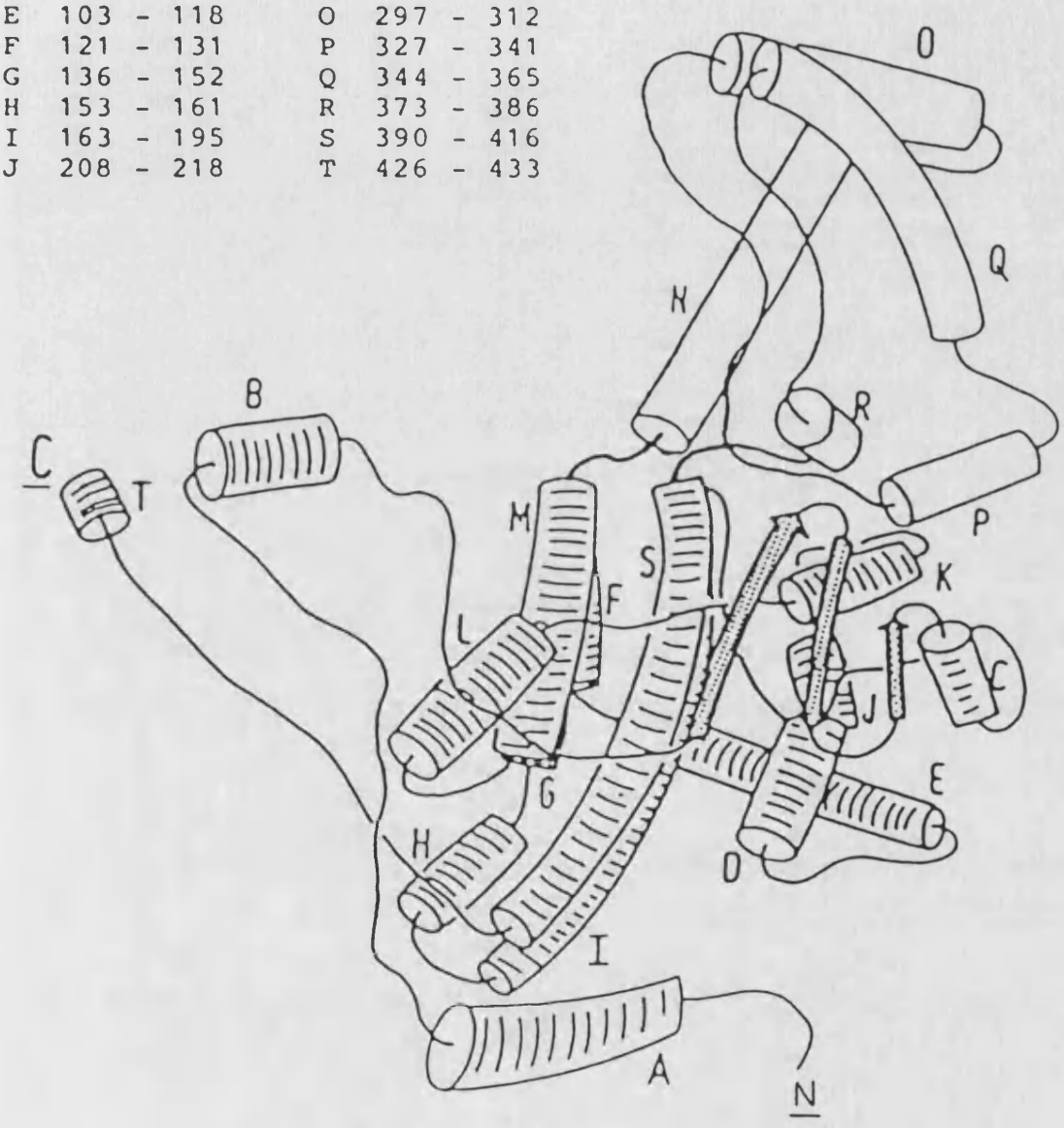
2.5 CITRATE SYNTHASE STRUCTURE

Studies on the crystallograhic structure of citrate synthase from pig heart (Remington et al., 1982; Wiegand et al., 1979) showed that the individual polypeptide chains were folded predominantly as helices. Thus the enzyme has been shown to contain 40 helices per dimer, packing tightly to form a globular molecule. Fig 2.3 represents the schematic structure of one monomeric unit of the dimeric enzyme. It has been shown that this monomer is divided structurally into two domains. The larger domain contains residues 1-274 (helices A to M) and residues 381-437 (helices S and

Fig. 2.3 Conformation of PHCS monomer as determined by X-ray crystallography

helical segments

A	5 - 29		K	221 - 236	
B	37 - 43		L	242 - 255	
C	70 - 78		M	257 - 271	
D	88 - 99		N	274 - 291	
E	103 - 118		O	297 - 312	
F	121 - 131		P	327 - 341	
G	136 - 152		Q	344 - 365	
H	153 - 161		R	373 - 386	
I	163 - 195		S	390 - 416	
J	208 - 218		T	426 - 433	



The table shows the amino acid residues at the beginning and end of each helical segment. (Remington et al., 1982).

T), while the smaller domain has residues 275-380 (helices N to R). The subunits are extensively interdigitated, with amino acid residues from one subunit contributing to the binding of citrate and CoA to the other subunit.

The structure of the enzyme has been described in three states (Wiegand et al., 1984; Remington et al., 1982). It has been proposed that binding of oxaloacetate to the "open" form of the enzymes induces a "closed" form in which the binding site for CoA is developed. A further "closed" form is found which has bound citrate. Lesk and Chothia (1984) and Chothia and Lesk (1985) have studies the conformational changes in response to changes in the state of ligation. The conformational forms of the enzyme were explained in terms of helix movements, the most pronounced being the movement of helix O on the outside of the small domain. This is in agreement with the proposal of Wiegand et al. (1984), who suggested that the small domain is more flexible than that the large one.

2.6 IMMUNOCHEMICAL STUDY OF CITRATE SYNTHASE

An antiserum raised against rat heart citrate synthase was able to cross-react with rat liver, kidney, brain and spleen enzymes, using Ouchterlony double diffusion technique (Moriyama and Srere, 1971). No reaction, however, was seen with PHCS, or CS from moth muscle, Azotobacter, or mango. Matsuoka and Srere (1973) used this antiserum for enzyme precipitation and in double immunodiffusion experiments, suggested that the rat kidney CS, heart CS and brain CS are immunologically identical.

Other antisera raised against yeast CS have proved useful for the precipitation of the translation products of mRNAs, to identify which mRNAs could be used to produce cDNA sequences coding for CS (Suissa et al., 1984; Alam et al., 1982).

Further experiments using double diffusion and immunoprecipitation were carried out on different proteolytic digestion mixtures of PHCS in an attempt to discover

which domain of PHCS had the greater antigenicity (Bloxham, et al., 1980). The relative impurities of the fragments used in this assay, however, may overrule the conclusion that the small domain is the primary antigenic domain.

Pullen et al. (1985) have reported an immunochemical investigation of interspecies diversity, using rabbit antibodies raised against PHCS. In a competition enzyme-linked immunosorbent assays, where plates were coated with pigeon breast CS, they showed the order of competition to be PHCS > pigeon breast CS > Bacillus megaterium CS > E. coli CS. The same order of reactivity was shown in enzyme inhibition experiments. Cross-reactivity was also studied by examining the ability of the rabbit anti-PHCS immunoglobulins to precipitate out enzyme activity from solution using Staphylococcus aureus protein A as a cross-linking agent. In this assay both the PHCS and E. coli CS tested were shown to be precipitated. This work shows conclusively that, in addition to species-specific determinants, the citrate synthases from pig heart, pigeon breast, B. megaterium and E. coli share common antigenic epitopes.

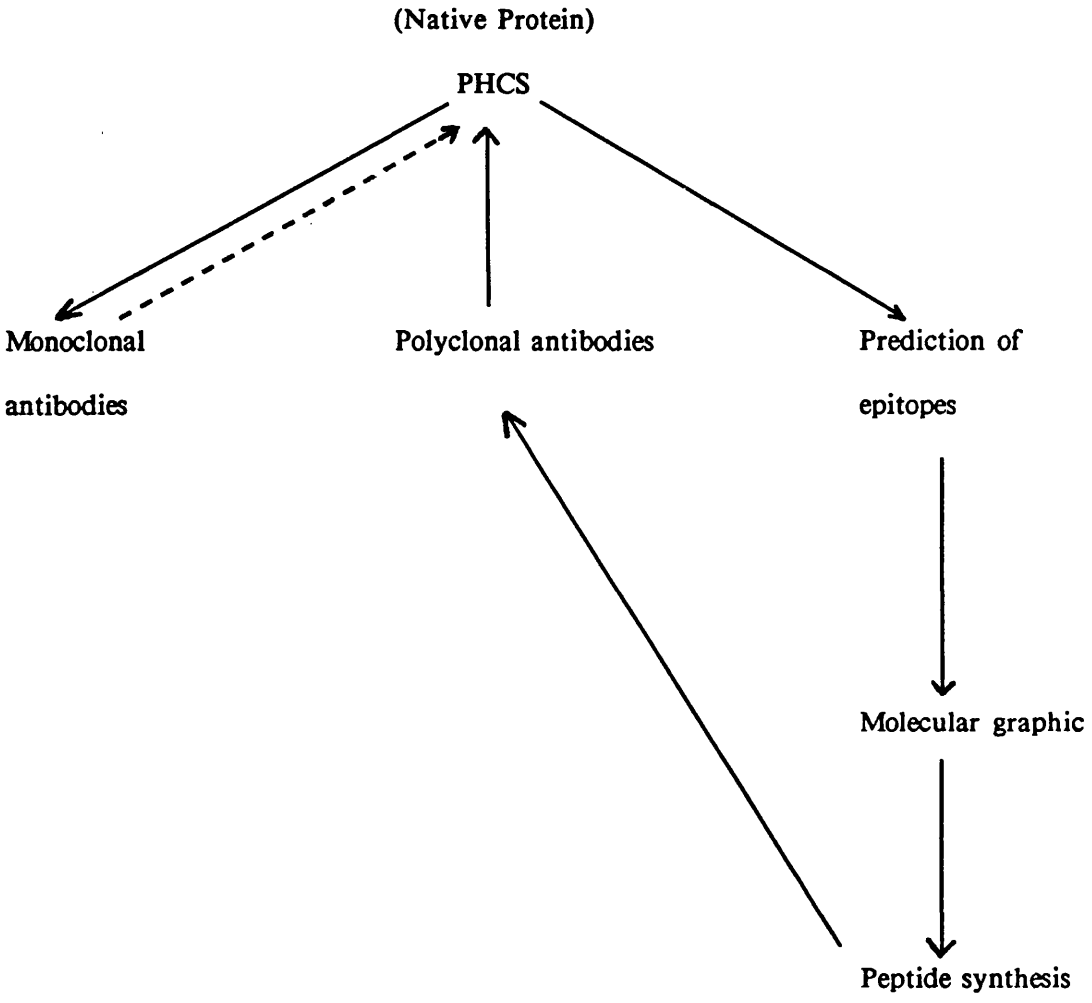
AIMS OF THIS STUDY

The aim of this work is to study the antigenicity of the enzyme pig heart citrate synthase (PHCS) by comparing experimentally identified epitopes, with epitopes identified using predictive methods. So, the work will include:

1. Prediction of potential epitopes.
2. Synthesis of selected peptides corresponding to potentially antigenic regions of PHCS.
3. Examination of their antigenicity with antibodies raised against native PHCS.
4. Use of these synthetic peptides as immunogens to raise serum antibodies.
5. Examination of the reactivity of anti-peptide sera against each peptide and native PHCS.
6. Determination of the ability of these anti-peptide sera to block the binding of MAb to native PHCS.
7. Comparison of rabbit-anti PHCS with these anti-peptide sera on immunoblots, against native PHCS and fragments of PHCS.
8. Study of the effect of anti-peptide sera on enzyme activity.

Fig. 2.4 schematically represents these aims.

Fig. 2.4 Locating major antigenic sites in PHCS using a combination of both experimental and predictive methods.



CHAPTER THREE MATERIALS AND METHODS

MATERIALS

3.1 REAGENTS

All chemicals used were of analytical grade, unless stated in text, and were purchased from either Sigma (UK) Ltd or BDH Ltd. Pig heart citrate synthase (PHCS) as a suspension in 2.2M $(NH4)_2SO_4$ solution pH 7.0, was obtained from Sigma. Polyclonal and monoclonal antibodies (MAb's) against PHCS were obtained from previous work in our laboratory.

All conjugated antibodies were obtained from Sigma; anti-rabbit IgG alkaline phosphatase and anti-mouse IgG alkaline phosphatase, were as suspension in 0.05M tris pH 8.0, and anti-rabbit IgG peroxidase was as a suspension in 0.01M phosphate buffered saline pH 7.4. Table 3-1 shows the amino acid derivatives and resin used for peptides synthesis which were purchased from Milligen (manufactured by Cambridge Research Biochemical (CRB), Ltd). N,N-dimethylformamide (DMF) (vacuum distilled prior to use), trifluoro acetic acid (TFA) (distilled from $KMnO_4$) and piperidine were from Aldrich Chemical Co. Ltd.

Table 3.1 The amio acid derivatives used in the peptides synthesis

amio acid	derivative used in peptide synthesis
Ala	Fmoc - Ala - OPf_p
Arg	Fmoc - Arg (Mtr) - OH
Cys	FmoC - Cys (Trt) - OPf_p
Asp	Fmoc - Asp (OBu^t) - OPf_p
Gln	Fmoc - Gln - OPf_p
Glu	Fmoc - Glu (OBu^t) - OPf_p
Gly	Fmoc - Gly - OPf_p
Leu	Fmoc - Leu - OPf_p
Lys	Fmoc - Lys (Boc) - OPf_p
Met	Fmoc - Met - OPf_p
pro	Fmoc - Pro - OPf_p
Ser	Fmoc - Ser (Bu^t) - ODhbt
Val	Fmoc - Val - OPf_p

The resin KA was used for the synthesis of peptides with a C-terminal carboxylic acid.

BOC: *tert*-butyloxycarbonyl.

Bu^t: *tert*-butyl.

Mtr: methoxytrimethylbenzene sulphonyl.

OBu^t: *tert*-butyl ester.

ODhbt: 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazol ester.

OPf_p: pentafluorophenyl ester.

3.2 SOLUTIONS

All solutions were prepared using glass-distilled or double-distilled water. Buffers, such as 0.05M carbonate pH 9.8 and 0.15M phosphate buffered saline (PBS) pH 7.2, were made as described by Hudson and Hay (1974) or Johnstone and Thorpe (1987).

METHODS

3.3 SYNTHESIS OF PEPTIDES

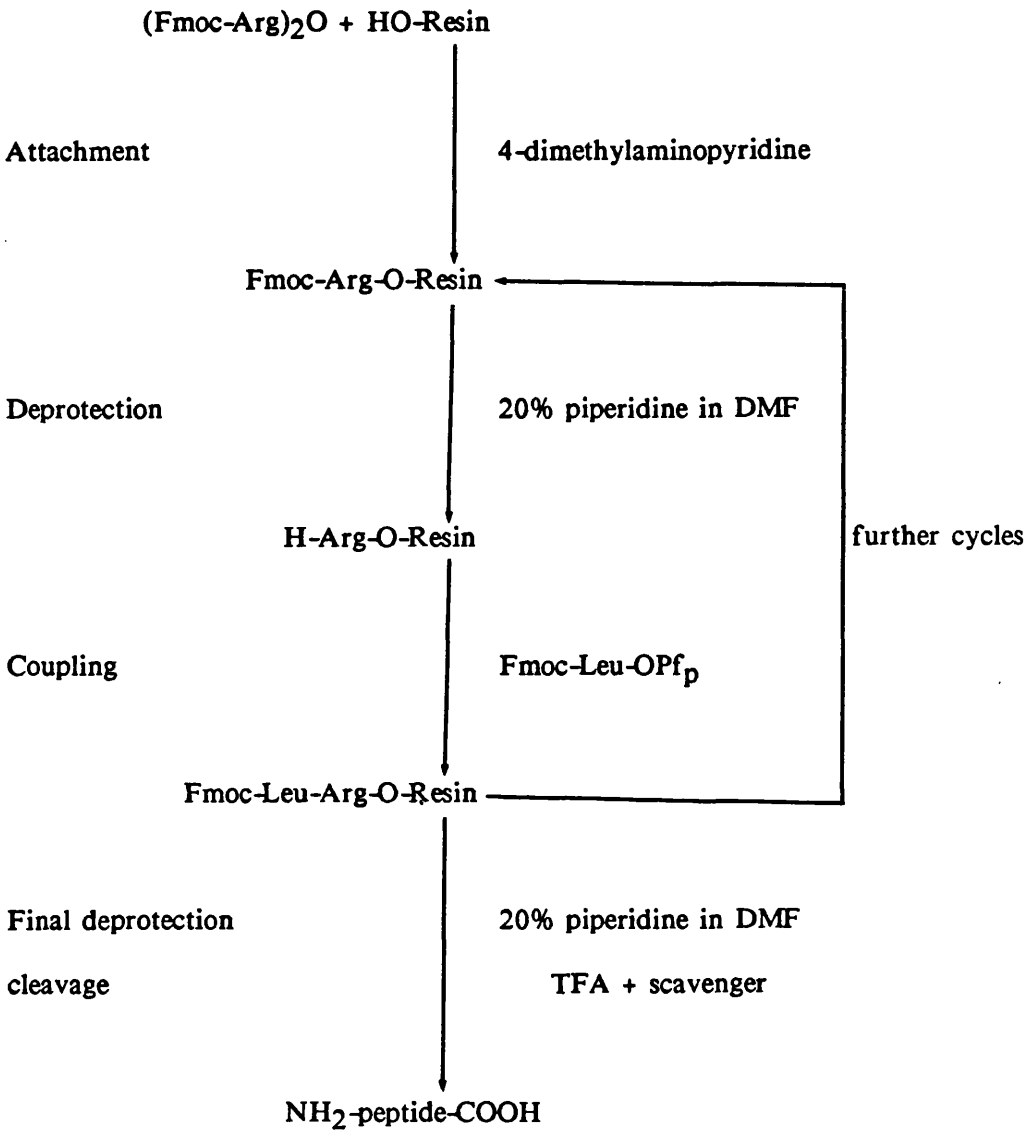
The solid-phase method of peptide synthesis was first introduced by Merrifield (1963). Recently an alternative chemistry (Fmoc polyamide active ester) has been developed and improved over the past few years by Sheppard and co-workers at Cambridge Research Biochemical (CRB).

a) Basis of the method

In solid-phase synthesis, peptide chains are assembled from the carboxy terminus, one amino acid at a time, towards the amino terminus.

The first (carboxy terminal) amino acid is attached by forming a chemical bond to an insoluble supporting resin via an acid chemical linking agent. In subsequent steps, Carboxy group activation is by an active ester, pentafluorophenyl (Pfp). The remaining amino acids are added one by one until the peptide assembly is complete. The amino (NH_2-) group of each amino acid is protected by substitution with a 9-fluorenylmethoxycarbonyl (Fmoc) group. This group is removed once coupling of the amino acid to the growing peptide is complete. The chemistry of initiation and one further cycle of solid-phase peptide synthesis is illustrated in Fig. 3.1.

Fig. 3.1 Schematic diagram of peptide synthesis



One cycle for synthesis of peptide PCS-1 as started with residue no. 302 arg followed by leu ... etc.

b) Coupling reaction and the test for complete coupling

The first (C-terminal) Fmoc amino acid residue was bound to the resin through the formation of an ester bond using 4-dimethylaminopyridine (DMAP) in DMF. Deprotection of the N-terminal amino group was achieved using 20% piperidine in DMF and then coupling of the following amino acid was achieved using the pentafluorophenyl (Pf_p) ester (activation) of the Fmoc amino acid. Elongation of the peptide chain was performed stepwise until the N-terminal amino acid was reached. 1.1 g of resin was used with a capacity of 0.1 meq/g and amio acid derivatives were used as 4 fold excess.

Pf_p esters were dissolved in the minimum volume (1-2ml) of DMF (with 1-hydroxybenzotriazole (HOBT) acting as a catalyst) before being loaded to the peptide synthesiser. The reaction was left for coupling for 25 min. and again deprotection using 20% piperidine in DMF was effected as shown in Fig. (3-1).

To check completion of the coupling reaction prior to deprotection, the "Kaiser test" was used. This test uses three reagents which are: 5% ninhydrin in ethanol, 400% phenol in ethanol and 2% 0.001M KCN in Pyridine; a blue colour is formed in the presence of free amino groups. A few grains of resin were taken from the top of the column just after the end of coupling time of each activated Fmoc amio acid loaded; this resin was washed on a small sintered glass filter with 10 ml each of DMF, DCM and ether then dried on an air line. The dried grains were then transferred to a tube (0.5x5 cm) and one drop each of the three Kaiser test reagents were added. The mixture was heated for 5 min at 100°C. Complete coupling is confirmed by a pale yellow colour. If the coupling is incomplete (as seen by the development of a blue colour), either the reaction time can be extended or fresh acylating agent can be added.

c) Cleavage of the assembled peptide

After complete assembly of the required peptide, final deprotection was carried out using 20% piperidine in DMF. The column was removed from the peptide synthesiser and the contents of the column (peptide-resin) were washed with 40ml each of t-amyl alcohol, acetic acid, t-amyl alcohol, and ether, after which the resin was dried using an air line. The required amount of peptide from the total amount of peptide-resin was weighed out and suspended in TFA (8.5 mg/ml) which contained a specific scavenger. It is necessary to add scavengers (5-10%) to the TFA to prevent side reactions with the released peptide. Commonly used scavengers are: water, phenol, ethylmythyl-sulphide, and ethanedithiol. The choice of scavenger depends upon the amio acid composition of the peptide. So in the presence of arg as in peptide PCS-1, the scavenger was 5% (w/v) phenol and in the presence of met in peptide PCS-2, 5% (v/v) ethylmethylsulphide was used as a scavenger. After cleavage for 18h in the case of PCS-1 and for 2-3h in the case of PCS-2, the resin was removed by filtration through a sintered funnel and the filtrate evaporated under reduced pressure to remove TFA. The peptide residue was then dissolved in 1-2 ml of double-distilled water and freeze-dried overnight. The dry peptide was collected, weighed and stored at -20°C.

3.4 AMINO ACID ANALYSIS

Amino acid analysis was used to confirm the composition of the synthetic peptides. Duplicate aliquots of 10 and 20μl (containing approximately 10 and 20μg peptide) of each synthetic peptide were hydrolysed at 110°C for 24 and 48h in 200μl of 6M HCl, in evacuated sealed tubes. Then, the hydrolysates were dried in a vacuum desiccator and resuspended in 25mM HCl (200μl) and subsequently loaded onto the amino acid analyser (Rank-Hilger chromaspek II).

3.5 PURIFICATION OF SYNTHETIC PEPTIDES

The crude peptides were desalted by gel filtration on a Bio-Gel P2 column (1x15 cm), eluting with 0.05M acetic acid. Peptide elution was monitored by absorbance at 230 nm. Then fractions containing the first absorbance peak were pooled and freeze-dried.

Peptides were further purified by fast protein liquid chromatography (FPLC) using a Pharmica FPLC system. Samples of peptide (dissolved in 10mM sodium acetate) were loaded onto a Mono S anion exchange column and the bound peptide was eluted with an NaCl gradient of 0.5M. (This Mono S column was used as a cation exchanger with $-CH_2-SO_3$ groups, so that the positively charged protein will bind). The separation was monitored by absorbance at 215 nm. The fractions containing the peptide peak were pooled and freeze-dried. Aliquots were taken for amino acid analysis. After purification, samples of synthetic peptides (1 mg/ml dissolved in acetonitrile) were tested for purity by HPLC on a Gilson reversed-phase C-18 column (0.46x25 cm) using an acetonitrile gradient (of 5% - 95% (v/v)). The purified material gave a single symmetrical peak as monitored by a uv detector at 214 nm.

3.6 PREPARATION OF ANTI-PEPTIDE ANTIBODIES

a) Extension of synthetic peptides and coupling to carrier protein

Two synthetic peptides, each of 15 amino acid residues (PCS-1 and PCS-2 corresponding to residues 288-302 and 76-90 of the PHCS sequence respectively), were synthesised as described in section 3.3, and before cleavage the peptides from the resins, both peptides were extended with two more amino acid residues using the same Fmoc technique. The additional amio acids were glycine, which was used as a spacer,

and cysteine. The cysteine residue was added to the amino terminus of the peptides to allow coupling to a protein carrier. Then, the extended synthetic peptides were cleaved from the resins as described in section 3.3.

The extended peptides were coupled to the carrier protein keyhole limpet hemocyanin (KLH). The carrier protein was activated with N-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) and subsequently coupled to the peptide through its cysteine residue (Liu et al., 1979; Green et al., 1982) as shown in Fig. 3-2.

For each peptide, 4 mg KLH in 250 μl of 10mM sodium phosphate buffer, pH 7.2, was reacted with 0.7 mg MBS (dissolved in 50-70 μl DMF), added dropwise with stirring for 30 min at room temperature. The reaction product, KLH-MB, was then passed through a Sephadex G-25 column (1x15 cm), equilibrated with 50mM sodium phosphate buffer, pH 6.0, to remove free MBS. KLH-MB was recovered by pooling fractions for the first peak of the column eluate (monitored by A_{280}) and then reacted with peptide (5mg dissolved in 1ml of PBS). The pH was adjusted to 7-7.5 and the reaction was stirred for 3h at room temperature. The mixture was stored at -20°C until required for immunisation.

b) Immunisation method

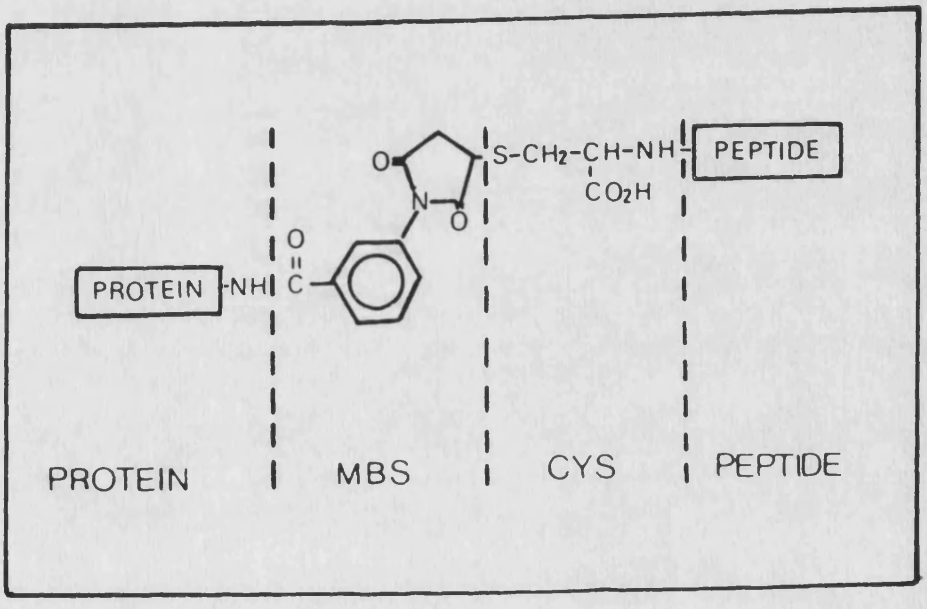
Six rabbits were used, two for each peptide-carrier conjugate and two for the carrier protein (KLH). Rabbits were injected intramuscularly with 1ml of immunogen (200μg of protein); injections were given in the hind limbs at multiple sites, according to the following schedule:

day (0) 1st injection: equal volumes of each immunogen and Complete Freund's Adjuvant were mixed and homogenised several times until an emulsion was obtained.

day (14) 2nd injection: as above but using Incomplete Freund's Adjuvant.

day (28) 3rd injection: aluminum potassium sulphate (alum) - precipitated immunogen. Each immuogen (0.5ml) was mixed with 0.2M alum (0.5ml) and 1M

Fig. 3.2 Peptide-Carrier conjugation using the MBS method



Carrier protein : Keyhole Limpet Hemocyanin

MBS : N-maleimidobenzoyl-N-hydoxysuccinimide ester

$NaHCO_3$ (0.22ml) was added, and the mixture was left for 15min at room temperature. Then the precipitate was collected by centrifugation at 300g (1500 rpm) for 15 min and the insoluble protein resuspended in PBS prior to immunisation.

After the initial course of injections, the rabbits were boosted with the same amount of alum-precipitated immunogen given at intervals of 1 and 2 weeks.

c) Preparation of Sera

Immunised rabbits, one and two weeks after an antigen boost (as mentioned above), were ear-bled for 10-15ml of blood each time. Five bleeds were taken and the fifth bleed was used in all the assays for this study.

Blood was left for 4-5h at room temperature, then overnight at 4°C for clotting. Clots were then removed by centrifugation, in a bench MSE centrifuge, at 2000g (4,000 rpm) for 15min and the serum was given a second centrifugation at 11000g (10,000 rpm) for 15min. Serum was collected and stored at -20°C in aliquots.

d) Immunoglobulin preparation from sera

An equal volume of saturated ammonium sulphate solution was added to rabbit serum. Following a 30min incubation at 4°C, with periodic stirring, the precipitate was collected by centrifugation at 5,000 g for 20 min. The pellet was redissolved in 1ml of PBS and dialysed against 20mM tris buffer, pH 8.0, at 4°C.

e) Protein determination

The concentration of protein was estimated spectrophotometrically (Layne, 1957). The absorbance of a suitably diluted protein solution was measured at both 260nm and 280nm against a buffer blank. The following formula was applied to calculate the

protein concentration in mg/ml;

protein concentration = $(1.55 \times A_{280} - 0.77 \times A_{260})$

3.7 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA reactions were carried out using flat-bottomed rigid polystyrene EIA strips (Titertek, Flow Lab.).

a. Direct ELISA

In this technique, the antigen is immobilised by direct attachment to a solid support (plastic sides of microtitre wells). Wells were coated with a solution of PHCS (10 μg protein/ml) in 0.05M sodium carbonate buffer, pH 9.8 (150 μl) for 18h at 4°C. Then the contents of the wells were tipped out and the plate blocked by incubation for 30min at 37°C with 150 μl of 1% (w/v) casein in PBS, followed by washing 4x5min with PBS containing 0.05% (v/v) Tween 20. Antibodies (150 μl), at different dilutions in PBS, were added and incubated for 2h at 37°C, followed by washing for 4x5min. The plates were then incubated for 2h at 37°C with 150 μl enzyme-antibody conjugate (as mentioned in section 3-1), diluted 1:1000 in 1% (w/v) casein/PBS. After washing for 4x5min, an enzyme substrate (150 μl) was added and the colour produced was read by using a "Titertek Uniskan" microtitre plate reader with a 405 nm filter.

b) Sandwich ELISA

Wells were coated with 150 μl rabbit IgG anti-citrate synthase (10 μg protein/ml in carbonate buffer) for 18h at 4°C, and were then blocked with 1% (w/v) casein in

PBS and washed. Antibody-coated wells were incubated with citrate synthase (10 μg/ml in PBS) for 2h at 37°C. Following this, the subsequent steps of the assay were carried out as described above.

c) Competitive ELISA

In this ELISA technique, all the steps explained above were used, except that the antibody was incubated with varying amount of antigen (as competitor) prior to transfer to the wells. That is, 200μl samples of antibody (20 μg/ml) were incubated with 200μl of antigen at various concentrations (0, 4, 10, 40, 100 μg protein /ml) for 1h at 37°C. Then duplicate 150μl samples of this mixture were added to the wells of a plate, previously coated with antigen. Then the subsequent steps of the assay were carried out as described above.

3.8 CHEMICAL FRAGMENTATION OF PHCS

a) Cleavage of asp-pro peptide bonds with mild acid was achieved according to Rittenhouse and Marcus (1984). PHCS (0.12 mg/ml) was incubated at 110°C with 15mM HCl in sealed tube. Termination of the reaction was accomplished by rapid cooling and freezing to - 70°C, and the digests were then freeze-dried overnight. The samples were reconstituted in water for running in SDS-PAGE (section 3.11).

b) CNBr cleavage at met was performed according to Gross (1967). PHCS (5mg/ml) was dialysed against 70% (v/v) formic acid at 4°C and solid CNBr (2mg/ml) was added to this solution. The reaction was carried out for 18-20h at room temperature in the dark, followed by dilution of the reaction mixture with 5-10 volumes of double-distilled water. Then the mixture was freeze-dried overnight and

reconstituted in water for separation on SDS-PAGE (section 3.11).

c) Hydroxylamine cleavage at asn-gly bonds was performed according to Bornstein (1970) and Bloxham et al. (1982).

Immediately prior to use, a reaction solution was prepared as follows: 1.8M hydroxylamine (3.48g) and 5.3M guanidine-HCl (14.32g) were dissolved in 13ml of water in an ice bath. 2.5ml of 12.5N NaOH was added slowly with vigorous stirring, followed by the addition of 5ml of 1M K_2CO_3. The pH of the solution was adjusted to 10.5 with additional NaOH and the volume was adjusted to 25ml.

1 volume of PHCS (10.6mg/ml) was mixed with 4 volumes of reaction solution and the mixture was incubated at 45°C. Aliquots was taken after incubation times of 7h, 14h, and 24h. The samples were immediately dialysed at 4°C overnight against 50mM tris-HCl, pH 8.0, containing 0.1% (w/v) SDS after which they were stored at -20°C for running in SDS-PAGE (section 3.11).

3-9 PREPARATION OF MAb-IMMUROSORBENT COLUMN

0.5g of cyanogen bromide activated sepharose-4B were swelled for 30min in 10ml of 1M HCl at room temperature and were then packed into a column of 1.5ml volume. The column was washed with 75ml of 1M HCl followed by 5ml of 0.2M $NaHCO_3$, pH 9.5.

Coupling of MAb was performed according to March et al. (1974). 1ml of MAb (which has been dialysed against 0.2M $NaHCO_3$,pH 9.5, at 4°C overnight) was added into the column (3.3mg MAb/ml of sepharose). Coupling was performed for 20h at 4°C. The beads were then washed with 5ml of 0.1M $NaHCO_3$,pH 10, and incubated with 2ml of 1% (w/v) casein in 0.2M $NaHCO_3$ for 18h at 4°C. Beads were washed with 10-15ml each of 0.1M sodium acetate,pH4.0, PBS, and 0.2M $NaHCO_3$,pH 9.5. Finally, the beads were suspended in PBS containing 0.02% (w/v) azide. The column

was stored and run at 4°C.

3.10 MAb IMMUNOSORBENT CHROMATOGRAPHY

Fragmented PHCS was reconstituted in PBS. For a column of 1.5ml gel volume, 300μg of PHCS fragments in 1ml of PBS was loaded, and left to equilibrate in the column for 6h. The column was then washed with 15ml each of PBS and 20mM tris-HCl, pH 8.0. The bound material was eluted with 3ml of 0.5M NH_4OH, pH 10.8, and freeze-dried. The freeze-dried eluants were reconstituted in water for analysis by SDS-PAGE (section 3-11). The column was re-equilibrated with PBS for storage.

ELECTROPHORESIS AND IMMUNOBLOTTING

3.11 GRADIENT SDS-PAGE

Electrophoresis was performed using the Atto slab gel apparatus with an acrylamide gradient of 8-25% on 2mm-thick polyacrylamide gels. A discontinuous tris-bicine buffer system containing 0-1% (w/v)SDS, as described by Johnstone and Thorpe (1987). The concentration of buffer in the resolving and stacking gels was 0.1M and 0.2M in the reservoir buffer.

The resolving gel mixtures were made up according to Table (3-2) and degased prior to addition of SDS and TEMED. 17ml of each gradient mixture was dispensed into a linear gradient maker connected to a peristaltic pump. The gel was poured at a flow rate of 3-5ml/min using a magnetic stirrer in the 25% (w/v) acrylamide well. Delivery to the mould was through a very thin tube pushed to the bottom-centre, of the gel and gradually retracted to keep it above the filling gel surface. On completion

of this, 1ml of water-saturated isobutanol was layered onto the gel and left overnight for polymerization, before the stacking gel was loaded.

The resolving gel surface was washed with water. The stacking gel mixture was made up according to the recipe in table (3.2) and degased prior to the addition of SDS and TEMED. Following insertion of the comb into the glass plates, the stacking gel was loaded with a pasteur pipette and allowed to set. Once this was performed, the comb was removed and the surface of the stacking gel washed with reservoir buffer to remove any unpolymerised acrylamide. Then, the gel was transferred to the running apparatus, with 400ml of buffer in the bottom chamber, and the whole assembly tilted to remove any air bubbles from under the gel. Then another 400ml of buffer was poured into the top chamber and the gel pre-run for 20min at 80V, prior to loading the samples.

The prepared samples (<100 μl) were loaded into the sample slots, using a 0-100μl Hamilton syringe. Electrophoresis was performed at 80V until the sample dye had entered the resolving gel, and finally at 100V until the dye front reached the bottom of the gel (approximately 12h).

3.12 SAMPLE PREPARATION FOR SDS-PAGE

The sample buffer was prepared by dissolving 1.51g of Tris-HCl and 20ml of glycerol in 35ml of water; the pH was adjusted to pH 6.75 with conc. HCl. Then, 4g SDS, 10ml 2-mercaptoethol and 4mg bromophenol blue were added and the volume made up to 100ml.

Freeze-dried samples were reconstituted with water (10-50μl) and an equal volume of sample buffer was added. The mixture was boiled for 2min prior to loading onto the gel. Two standard proteins mixtures were used: high molecular weight (β-galactosidase,116K; BSA,66K; glyceraldehyde-3-phosphate dehydrogenase,36K; carbonic

Table 3.2: Composition of resolving and stacking gels used in SDS-PAGE of PHCS peptide fragments

Chemical	Volume (ml)		
	8%	25%	stacking 3.75%
Acrylamide/bis-acrylamide (47.6% : 2.4%) (w/v)	4.8	15.0	1.5
1M Tris, 1Mbicine (pH 8.2)	3.0	3.0	2.0
water	21.2	8.3	15.3
10% SDS (w/v)	0.3	0.3	0.2
Sucrose (vol.=2.7ml)	-	4.5g	-
1.5% NH_4 persulphate (w/v)	0.7	0.7	1.0
TEMED	10μl	10μl	15μ
TOTAL	30	30	20

anhydrase,29K and trypsin inhibitor,20K) and low molecular weight (Myoglobin, 16.9K; myoglobin "fragment I+II", 14.4K; myoglobin "fragment I", 8.1K; myoglobin "fragment II", 6.2K; and myogloblin "fragment III", 2.5K). These were also dissolved and boiled in the sample buffer, so that 5μl of the molecular weight standard gave approximately 5μg of protein per band. Up to 100μl of sample was loaded per track (0.9mm diameter, 1.8mm depth). From these known mol. wts. proteins standard, a graph of Log_{10}%T versus Log_{10} mol.wt. was plotted.

Log_{10}%T was calculated from the formula:

$Log_{10}\%T = Log_{10}\%[(D \times \Delta 1\%) + \text{initial gel concentration}]$

where D: is the distance travelled through the gel,

and $\Delta 1\% = \frac{\text{change in gel concentration}}{\text{gel length}}$

3.13 GEL STAINING

Before staining for protein, the gels were soaked for 1-2h at room temperature with several changes of fixing solution [25% (v/v) butanol, 10% (v/v) acetic acid and 65% (v/v) water]. The gels were then stained by incubation for 1-2h in 0.025% (w/v) Coomassie blue-R (in 7% (v/v) acetic acid) on a shaker at room temperature.

Destaining was achieved using several changes of destaining solution [7% (w/v) acetic acid], until a clear background was obtained on the gels.

3.14 ELECTROPHORETIC TRANSFER OF PROTEINS TO NITROCELLULOSE

The blotting apparatus used was similar to the Bio-Rad Trans-Blot^{TM} cell, but had 2 graphite plates (175mm x 222mm x 10mm) as the electrodes.

After running as described in section 3.11 the resolving gel was removed carefully

from the apparatus and tracks were marked with a scalpel. The gel was rinsed in distilled water and washed for 15min in blotting buffer (2mM sodium acetate, 5mM MOPS, 20% (v/v) ethanol, pH 7.5). Nitrocellulose sheets (from Schleicher and Schuell, with pore size of 0.2μm) were cut to the same dimensions as the gel and was soaked for 10min in the blotting buffer. Two sheets of Whatman 3MM filter paper were cut and saturated with blotting buffer.

The gel was placed on one sheet of filter paper, on a Bio-Rad Transblot sandwich pad, and the nitrocellulose sheet was carefully placed over the gel and covered with the second sheet of filter paper. The second sandwich pad was placed over this, and the assembled cassette was immersed in a tank containing the blotting buffer, with the gel towards the cathode and the nitrocellulose towards the anode. During the assembly of these components, care was taken to avoid any air bubbles between successive layers of components, since air bubbles will disrupt the elctrical field and alter the pattern of protein migration from the gel onto the nitrocellulose sheet. The electrophoretic transfer was carried out for 12h at 10 V/6mA, with stirring, and using a water cooling coil in the centre of the apparatus.

3.15 AMIDO BLACK STAINING OF NITROCELLULOSE BOUND PROTEINS

Nitrocellulose sheets with blotted protein bands were incubated for 5min with 1% (w/v) naphtol blue-black in methanol/acetic acid/water (5:1:4). Destaining was performed in methanol/acetic acid/water (5:1:4).

3.16 IMMUNOBLOTTING OF NITROCELLULOSE BOUND PROTEIN

Following electrophoretic transfer, nitrocellulose strips were incubated for 4-5h at room temperature with 1% (w/v) casein in PBS to block non-specific protein binding. The strips were kept in separate containers for all further incubations.

The antisera used were diluted 1/50 in 1% (w/v) casein/PBS. 10ml of antibody solution were used in each container, and these were incubated for 4h on a shaker at room temperature. Strips were washed for 4x10min in 1% (w/v) casein/PBS and incubated with the conjugate (horse radish peroxdase) diluted 1:1000 in 1% (w/v) casein/PBS for 2h at room temperature, with shaking. The strips were then washed for 4x10 min with PBS (azide free).

The colour was developed using: 0.02% (w/v) 3-amino-9-ethylcarbazole, 5% (v/v) dimethylformamide, 0.00025% (v/v) thiomersal, 0.03% (v/v) H_2O_2 in 0.05M sodium acetate buffer, pH 5.0, as substrate. Development was continued until distinct bands were visible; the reaction was then stopped by washing the strips with water.

3.17 DOT-BLOT IMMUNOSTAIN

Sections of nitrocellulose sheets (40x80mm) were cut, and spotted in duplicate with 2μl aliquots of serial dilutions (0.1, 0.2, 1, 10 μg protein/ml in PBS) of PHCS. After drying at 45°C, the nitrocellulose sheets were blocked by incubating at room temperature for 1h with 1% (w/v) casein/PBS. The nitrocellulose sheets were then incubated with antisera (diluted 1/100 and 1/50 in PBS) at room temperature for 2h. Five such nitrocellulose sheets were used, one being incubated with anti-PHCS sera (1/100), and two being incubated with anti-peptide PCS-1 sera and anti-peptide PCS-2 sera of different dilution (1/50 and 1/100). Following this, the subsequent steps for the assay were carried out as described above in section 3.16.

3.18 CITRATE SYNTHASE ASSAY

Acetyl CoA was prepared by the acetylation of CoA with acetic anhydride, as described by Stadtman (1957). 10mg of CoASH (free acid) were dissolved in 1ml of water, cooled to 0°C, and 1M $KHCO_3$ (200μl) was added to bring the pH to pH 7.5. Then 1M acetic anhydride (100μl) was added and the mixture allowed to stand for 10min. The acetylation was tested by reaction with 5,5-dithiobis (2-nitrobenzoic acid) (DTNB); 10μl of 10mM DTNB were added to 1ml of buffer solution containing 20μl of the acetyl-CoA preparation and the free CoA concentration was calculated from the A_{412}.

Citrate synthase was assayed spectrophotometrically by the method of Srere et al. (1963). All assays were carried out at 25°C. Unless otherwise stated the assay mixture contained 20μl of 10mM acetyl-CoA, 20μl of 10mM oxaloacetate, 10μl of 10mM DTNB and 10μl of enzyme solution in a total volume of 1ml of buffer (20mM tris-HCl, pH 8.0/1mM EDTA). The reaction was monitored by measuring the increase in absorbance at 412nm due to the reaction of the CoA produced with DTNB (molar absorbance coefficient of the thionitrobenzoate at 412nm = 13,600 L/mol/cm, at pH 8.0 and 25°C).

a) Effect of anti sera on CS activity

Pure enzyme was diluted, so that a 10μl sample would give an absorbance change of approximately 0.04 units/min in the standard CS assay. 10μl of samples of such enzyme were incubated with a range of concentrations of antisera (anti-peptide sera, anti PHCS sera and normal rabbit sera) or of IgG's of those sera (equivalent to 0-150μg of IgG) in 1ml of standard CS assay mixtures lacking oxaloacetate. After 5 min at 25°C, oxaloacetate was added and the CS activity assayed as normal.

b) Effect of antisera and protein A

Only the binding of antibodies to determinants at or near the active site is likely to affect enzyme activity. However, in order to detect all antibody binding to PHCS, regardless of their fine specificity, an insoluble preparation of Staphylaoccus aureus protein A was used. This membrane-protein binds IgG via a site in its Fc region and so will precipitate all antigen-antibody complexes. Hence such complexes can be detected regardless of their effects on CS activity.

Protein A was purchased from Sigma company as insoluble, crude lyophilised cells of S. aureus with a quoted binding capacity of 0.061mg of IgG/mg of solid. Since those crude cells have endogenous CS activity, which will affect the assay measurements, the insoluble protein A was washed four times in incubating buffer for 15min that is, until only a negligible amount of CS activity remained. To investigation the binding of antisera or IgG's to CS, method (a) was modified by replacing 300μl of the buffer with a suspension of the insoluble protein A (5mg/assay). After standing for 30min at 4°C, the tubes were centrifuged for 5min at 2,000g to pellet the insoluble protein A -antibody- enzyme complexes and the supernatant was assayed for residual CS activity.

CHAPTER FOUR SELECTION AND SYNTHESIS OF PEPTIDES

4.1 PREDICTION OF THE EPITOPES OF PHCS

A variety of predictive methods have been used to determine potential antigenic and immunogenic peptide sequences of PHCS. Some of these methods are based on amino acid sequence and others on crystallographic data. These methods are:

a) Hydrophilicity

This method was first described by Hopp and Woods (1981).

From the solvent parameters of Levitt (1976), each of the 20 amino acids commonly found in proteins is assigned a hydrophilicity value. The values used are shown in Table 4-1. A hydrophilicity profile is achieved by repetitively averaging these values for overlapping sets of 6 residues along the chain and plotting them versus the residue number of the leading residue.

The hydrophilicity profile for PHCS was performed by Garforth (1985). In this work he adapted a program written in Hewlett-Packard BASIC, for use on a BBC micro-computer. The profile obtained is shown in Fig. 4.1.

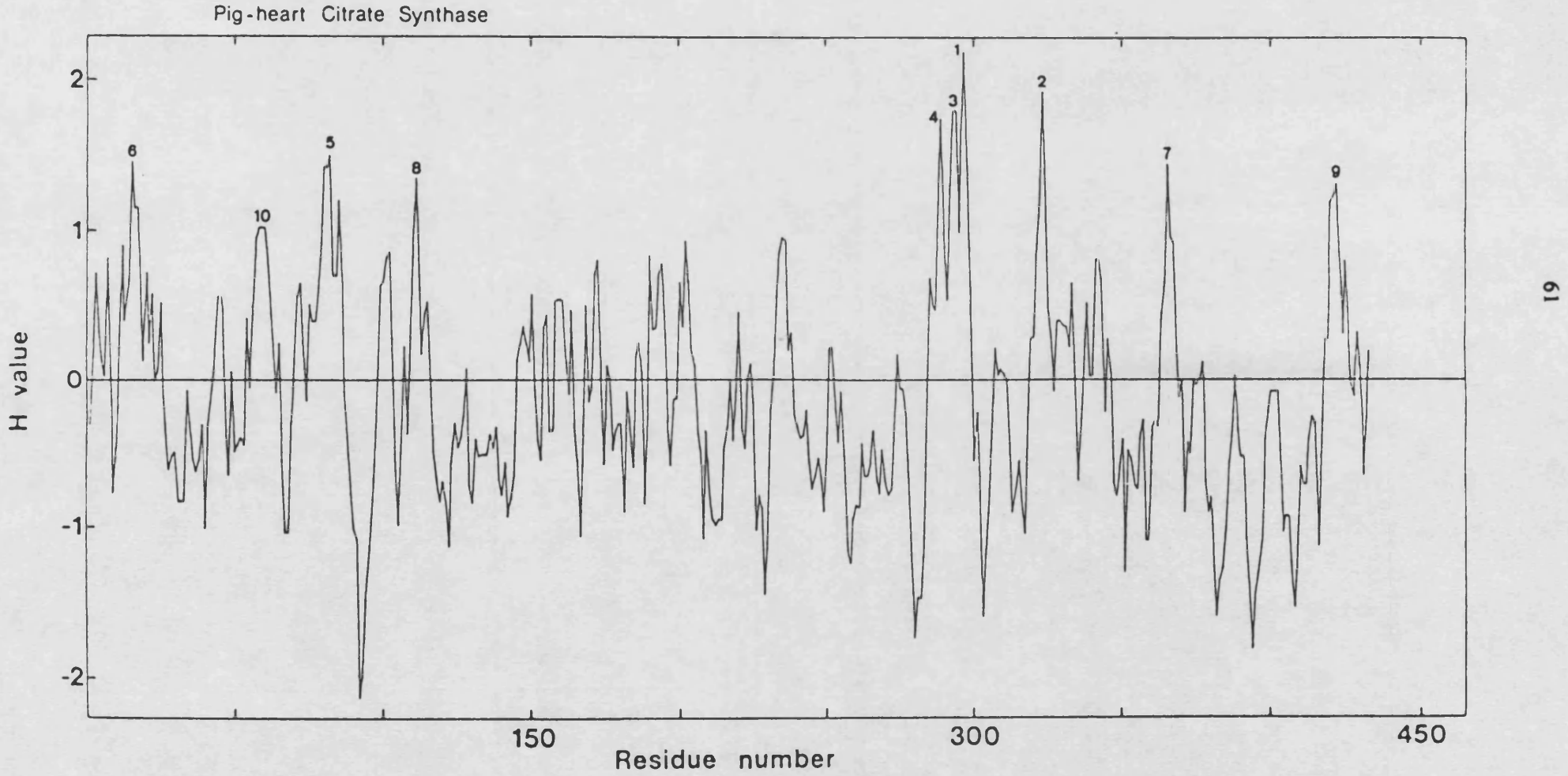
It can be seen from the results shown in Fig. 4.1 that the top five peaks of hydrophilicity occur at residues: 297-302, 323-328, 293-299, 289-294, and 82-87. However, it should be noted that, according to Hopp and Woods, only the highest peak of hydrophilicity was invariably found to correlate with antigenic sites in the model protein system they used (including, swMb; chicken lysozyme; horse heart cytochrome c). For PHCS, the highest peak of hydrophilicity occurs between residues 297-302.

Table 4.1 Hydrophilicity values assigned to individual amino acids

Amino acid	Hydrophilicity value*
Arginine	3.0
Aspartic acid	3.0
Glutamic acid	3.0
Lysine	3.0
Serine	0.3
Asparagine	0.2
Glutamine	0.2
Glycine	0.0
Proline	0.0
Threonine	-0.4
Alanine	-0.5
Histidine	-0.5
Cysteine	-1.0
Methionine	-1.3
Valine	-1.5
Isoleucine	-1.8
Leucine	-1.8
Tyrosine	-2.3
Phenylalanine	-2.5
Tryptophan	-3.4

* Based on the solvent parameter values of Levitt (1976).

Fig. 4.1 Hydrophilic profile



b) Composite surface profile

This method was described by Parker et al. (1986).

Each of the 20 amino acids commonly found in proteins was assigned a "surface profile value". The surface profile values were arbitrarily assigned on a scale of 0-100, and are a composite of HPLC hydrophilicity indices, accessibility and flexibility values. A composite surface profile is achieved by repetitively averaging these values for overlapping sets of 7 residues along the chain, and plotting them versus the fourth residue number of each set of 7.

The composite surface profile for PHCS was performed by Dr. J. M. R. Parker, and is shown in Fig. 4-2.

It can be seen that the top five peaks of composite surface profile occur at residues: 236-246, 325-332, 78-90, 288-302 and 363-372.

c) Mobility

Use of X-ray crystallography gives not only the precise atomic coordinates, but also atomic temperature factor (B values). The temperature factor represents the mean-square displacement of each atom, and when plotted against residue number gives a graphic image of the degree of mobility existing along the polypeptide chain, as described in section 1.3(d).

The B-values obtained for the atoms in PHCS were determined by Remington et al. (1982). The mobility profile is shown in Fig. 4.3.

From the results shown in Fig. 4.3, it can be seen that three major peaks of mobility occur at residues: 81-85, 285-302 and 1-5.

Fig. 4.2 Surface profile for pig heart citrate synthase

(J Parker, Edmonton)

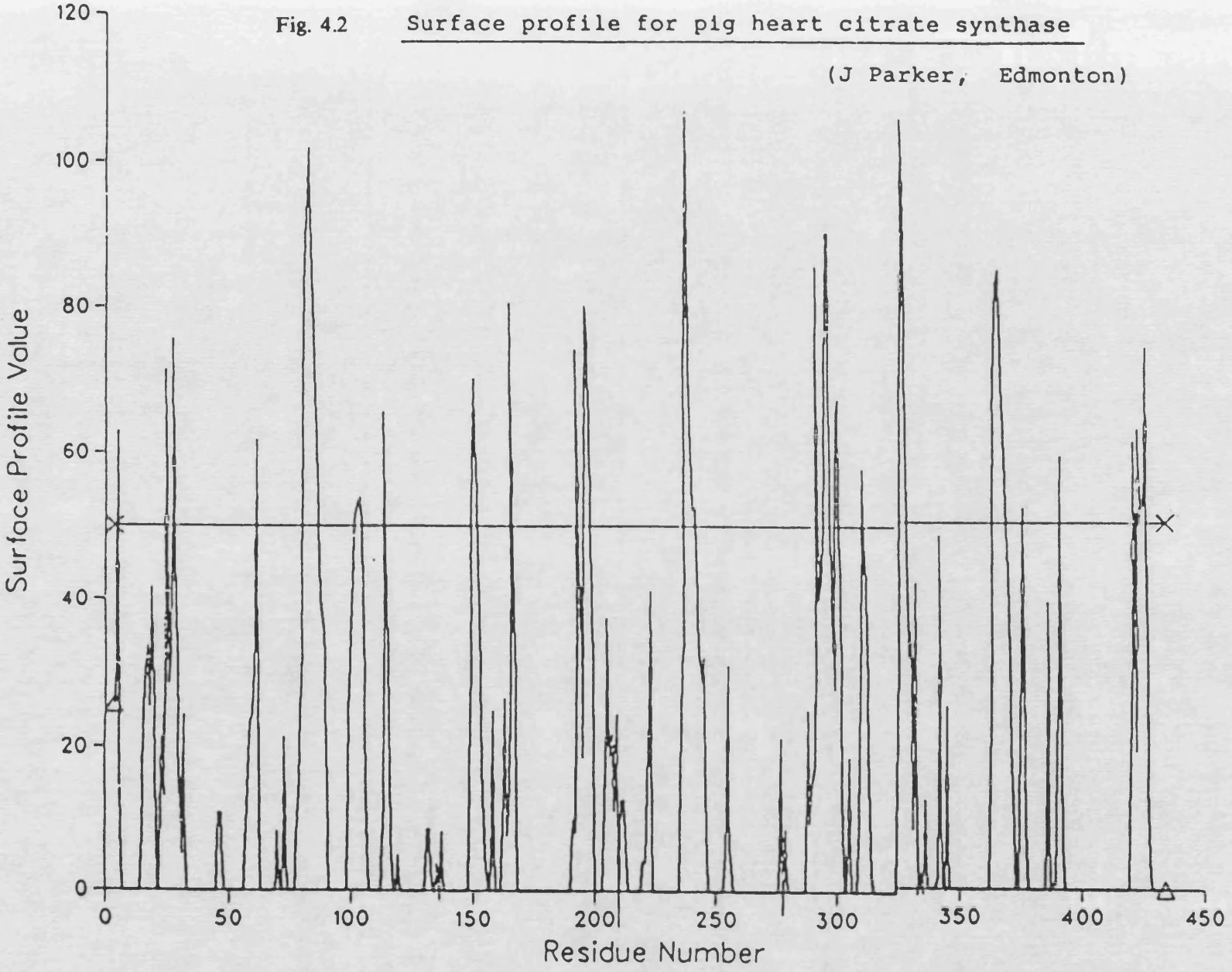
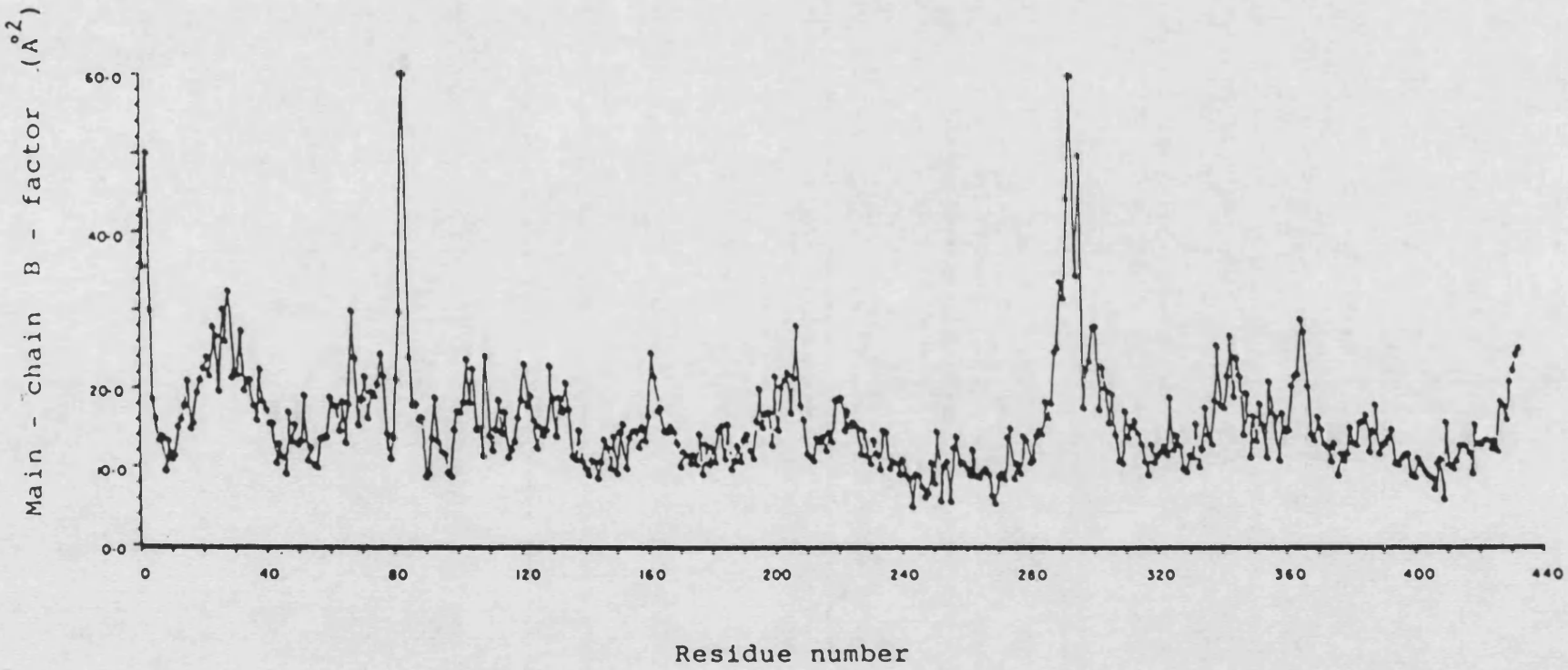


Fig. 4.3 Main-chain B-factors for citrate synthase



d) Protrusion indices

This method was first proposed by Thornton et al. (1986) and has been described in section 1.3(c). Protrusion indices have been calculated for PHCS from its X-ray crystallographic atomic coordinates. These PI values are calculated values for each amino acid of the PHCS and are a measure of the extent to which a residue protrudes out from the surface of the molecule. The data used were provided by Dr. J. M. Thornton (Birkbeck College, University of London).

Protrusion index profiles were drawn by plotting the PI value of each amino acid against its residue number shown in Fig. 4-4.

A further protrusion index profile was obtained by repetitively averaging PI values for overlapping sets of 6 residues along the chain and plotting them versus the residue number of the last residue in the set. These data are presented in Fig. 4-5, from which it can be seen that the most protrusive regions contain residues 288-305, 431-437, 22-33, and 194-207.

These data are for the native, dimeric enzyme PHCS. However, it is possible to calculate the PI values of each amino acid for one subunit of the enzyme by using the same method. But, the protrusive regions found in this case are different from those in the dimer, as shown in Table 4-2.

Since this method depends on the shape of the molecule, and the enzyme PHCS exists in its native state as a dimer with two identical subunits, therefore the prediction of epitopes would be more appropriate when it is based upon the native dimer conformation.

Fig. 4.4 Protrusion index profile of PHCS

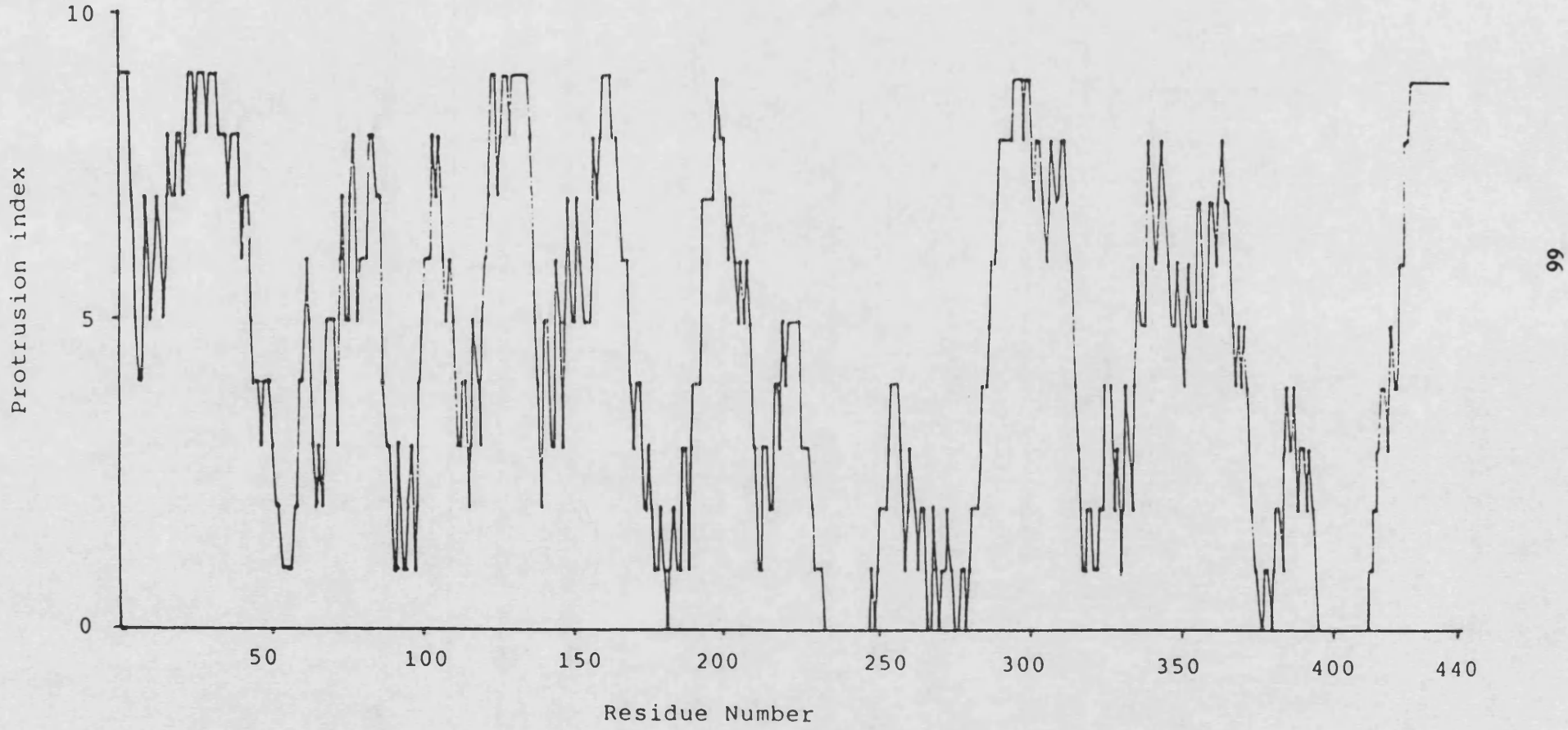


Fig. 4.5 Averaged protrusion index profile of PHCS

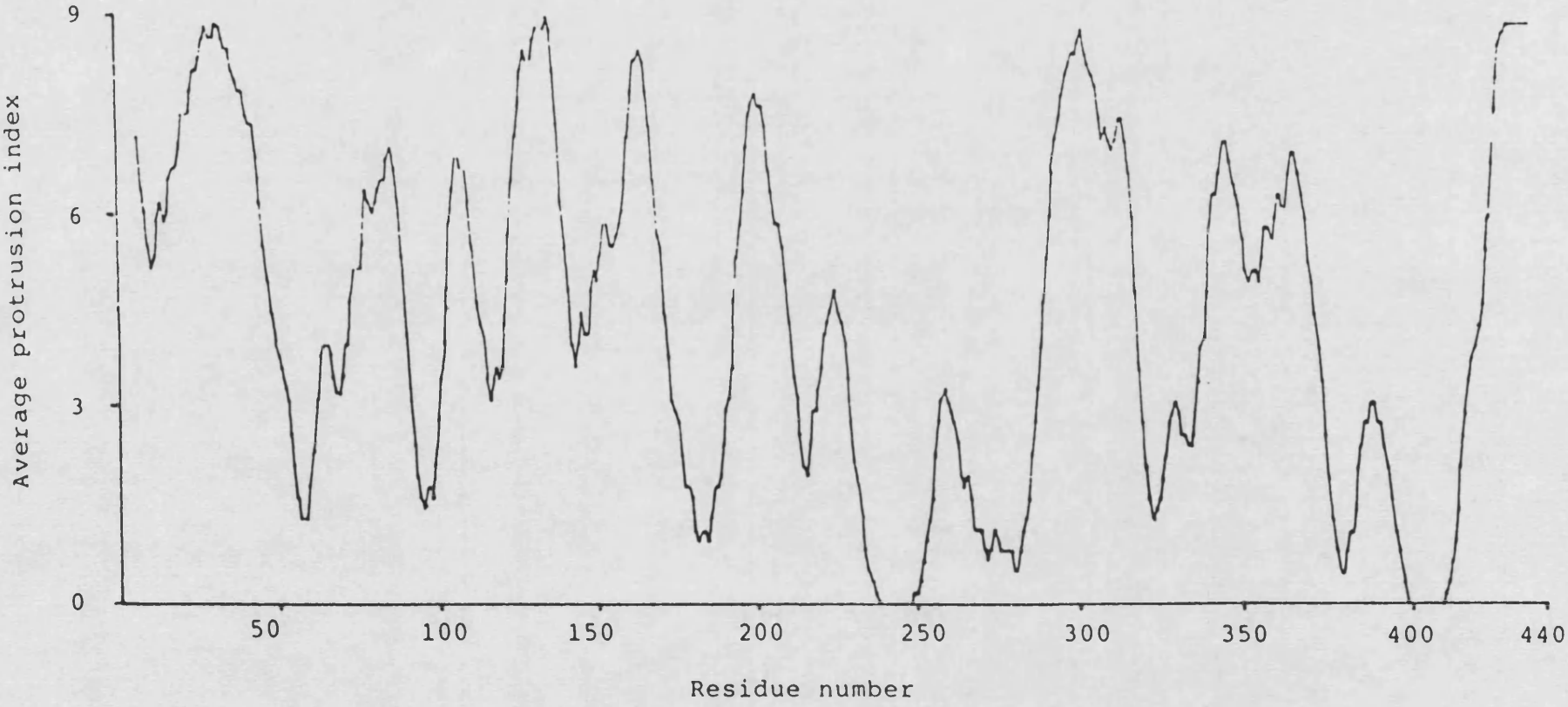


Table 4.2 Comparison between the protrusive regions of monomer and dimer enzyme PHCS

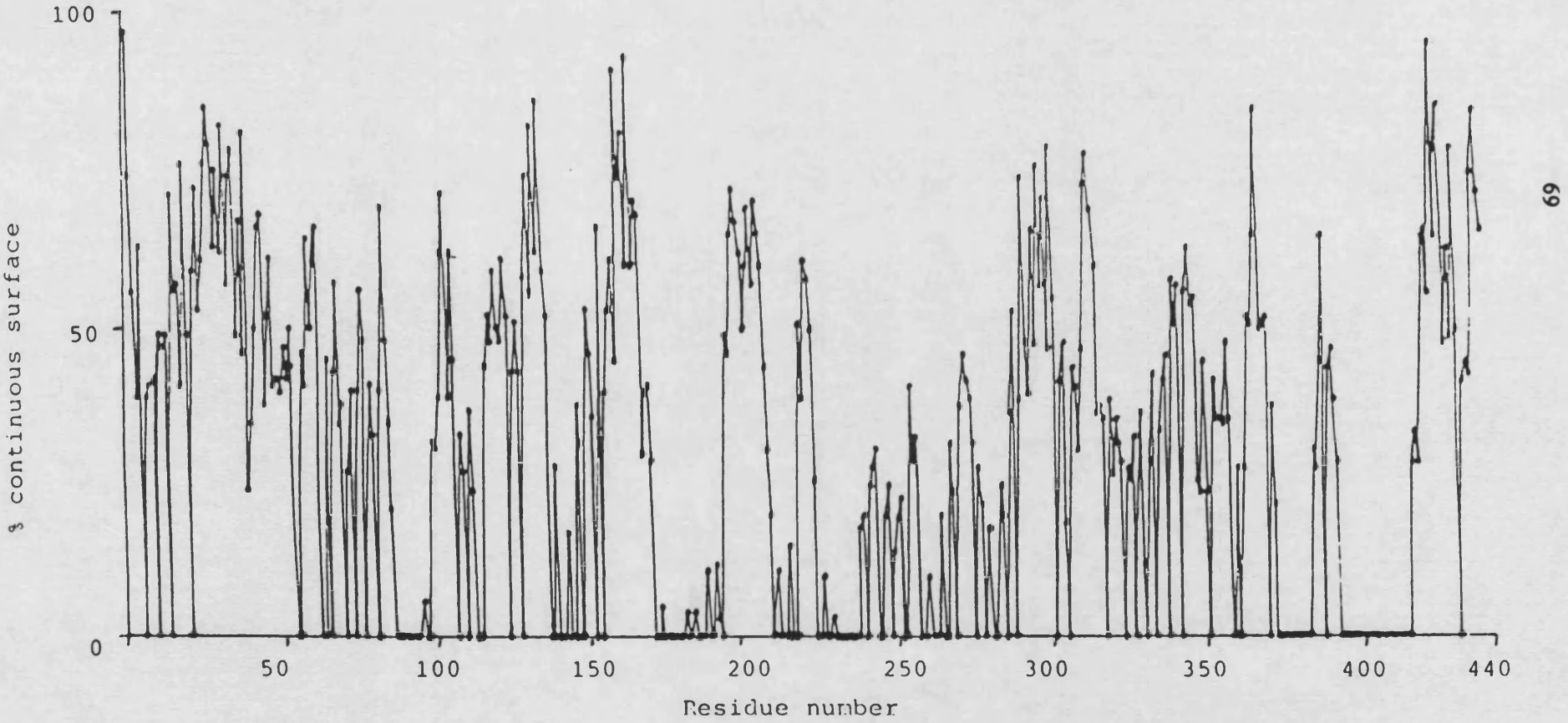
dimer	average protrusion	monomer	average protrusion
288 - 305	8.89	423 - 437	8.95
431 - 437	8.75	122 - 137	8.51
22 - 33	8.69	289 - 301	8.41
194 - 207	8.64	15 - 44	7.10

e) Surface continuousness

This work was first described by Barlow et al. (1986). The method used involves centering a sphere of radius 6Å on each surface atom of the protein, and calculating the proportion (F) of the centres of other surface atoms enclosed by the sphere which belong to residues local in the amino acid sequence. Surface atoms were defined as those with contact areas > $2Å^2$. Local surface atoms were defined as those ±3 residues distant in the sequence of the residues under consideration. Residues which contain no surface atoms were assigned a value of F = 0.

The "continuous surface" profile was performed by Dr. J. M. Thornton. These data are presented in Fig. 4.6. From these data, using a cut off at 50% "continuousness", it was predicted that the most likely continuous antigenic sites would contain residues: 418-424, 201-206, 159-166, 129-136 and 25-35.

Fig. 4.6 Histogram of % continuous surface (F) for residues in PHCS



f) Conclusion

In this section five different predictive approaches have been applied to compute the likely antigenic sites present on PHCS.

The major antigenic sites predicted by each method are shown on Table 4-3. Predictions made by methods a, b, c and d all suggest that a region contained within residues 288-302 is likely to be an antigenic site. Results from method (e) do not predict this region to contain a continuous epitope as residues 288, 290, 291, 294, 298, 301 and 302 show less than 50% "continuousness". Therefore an epitope is strongly predicted to occur between residues 288-302, but it is likely to be partly discontinuous. One further discontinuous epitope (residues 76-90) is predicted by methods a, b and c which is in a different region of the PHCS molecule, as shown in Fig. 4.7.

So, in this study these two peptides (288-302 and 76-90) have been synthesised, purified and used as both antigens and immunogens as will be discussed in the following sections.

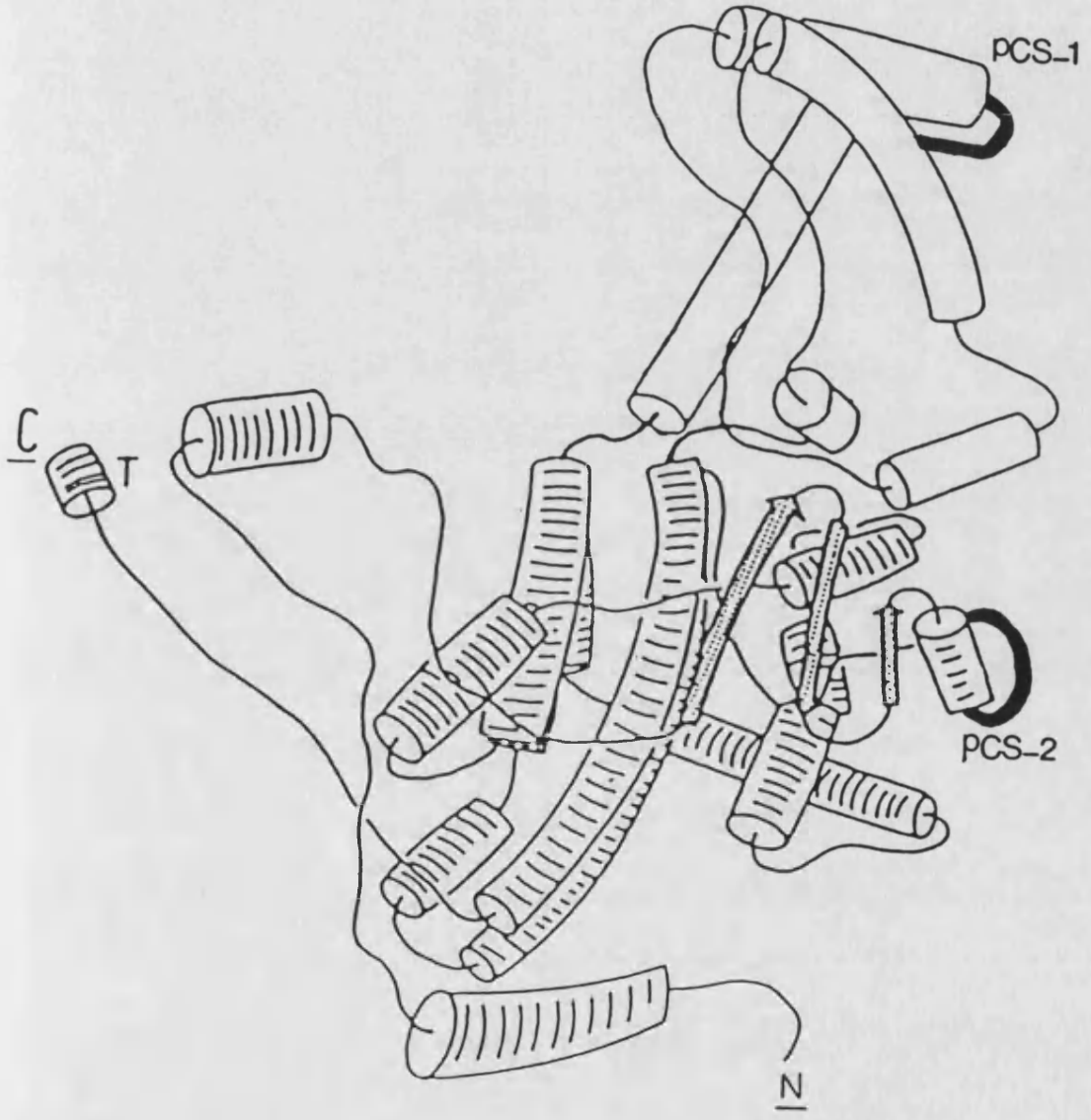
4.2 SYNTHESIS OF PEPTIDES

As pointed out in section (4.1), peptides selected for synthesis were those which were determined by more than one predictive method and located in distinct regions of the surface of the native structure as shown by molecular graphics diagram Fig. 4.7. Therefore two peptides have been selected of 15 amino acid residues each: (PCS-1) which represented the peptide at region 288-302 and (PCS-2) representing the peptide at region 76-90. The sequences of these peptides are shown in Fig. 4.8. Peptides were synthesised as described in section 3.3 by the solid-phase method using Fmoc protecting groups. Peptide PCS-1 was synthesised, starting from the Carboxy-terminal

Table 4.3 Summary of the predicted epitopes of PHCS

A) Hydrophilicity	(1)	297-302
	(2)	323-328
	(3)	293-299
	(4)	284-294
	(5)	82- 87
B) Composite surface profile	(1)	236-246
	(2)	325-332
	(3)	78- 90
	(4)	288-302
	(5)	363-372
C) Mobility	(1)	81- 85
	(2)	285-302
	(3)	1- 5
D) Protrusion	(1)	288-305
	(2)	431-437
	(3)	22- 33
	(4)	194-207
E) Surface Continuogram	(1)	418-424
	(2)	201-206
	(3)	159-166
	(4)	129-136
	(5)	25- 35

Fig. 4.7 PHCS monomer showing the position of synthetic peptides



peptide PCS-1 at region 288-302

peptide PCS-2 at region 76-90

Fig. 4.8 The sequences of peptides PCS-1 and PCS-2

PCS-1 (288-302)

288 302

leu - gln - lys - glu - val - gly - lys - asp - val - ser - asp - glu - lys - leu -arg

PCS-2 (76-90)

76 90

lys - met - leu - pro - lys - ala - lys - gly - gly - glu - glu - pro - leu - pro -glu

amino acid arg (residue no. 302) backward to the amino acid (no. 288) leu. Also, PCS-2 was synthesised from the amino acid residue no. 90 (glu) backward to residue no. 76 (lys). After each amino acid residue was added to the growing peptide, a colour test was performed to confirm the coupling efficiency, while amino acid analysis was performed at the completion of each synthesis.

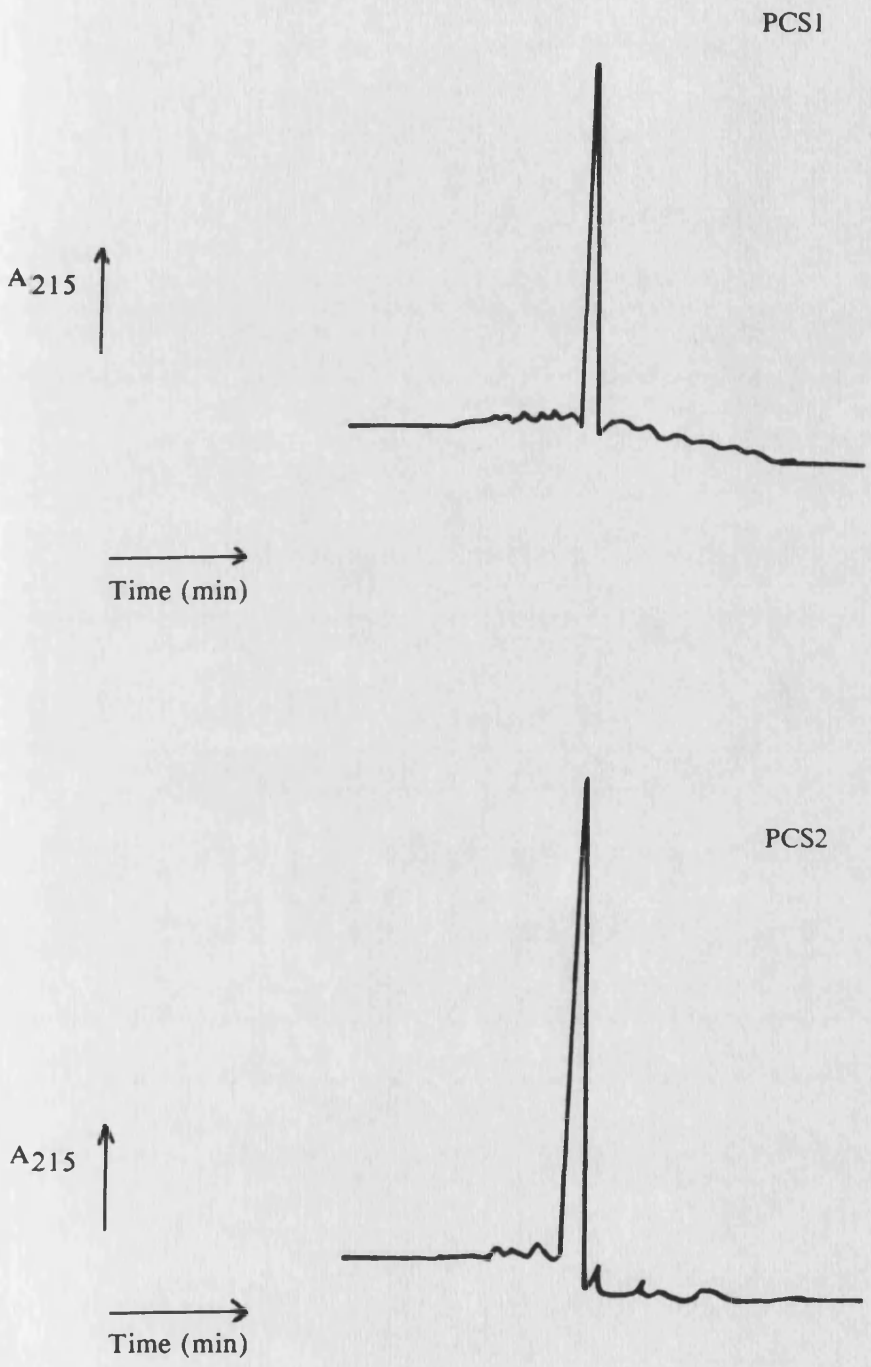
4.3 PURIFICATION OF SYNTHETIC PEPTIDES

After the two peptides had been synthesised, the purity of each peptide was assessed by HPLC as described in section 3.5. Samples from each peptides were analysed by HPLC at a concentration of 1mg/ml (in 5% acetonitrile). Fig. 4.9 shows a distinct and clear peak corresponding to pure peptide. Furthermore, purity of peptides was also identified using chromatography on the Pharmacia FPLC system, as described in section 3.5. After desalting the synthetic peptides through a column at Biol Gel P_2 as explained in section 3-5, the peptides were dissolved in 10mM NaAc pH 5.0 and injected through a 500μl loop to the FPLC system containing a Mono S anion exchange column. The bound peptide was then eluted with an 0.5M NaCl gradient. After the end of the run, fractions corresponding to peptide peak as shown in Fig. 4.10 were pooled and freeze-dried overnight. The pure peptides were stored at -20°C.

4.4 AMINO ACID ANALYSIS

Amino acid analysis as described in section 3.4 was determined for samples of both peptides at the completion of synthesis. Samples in duplicate were hydrolysed in 6M HCl, in evacuated tubes at 110°C for 24h and 48h. Then, the solution was evaporated to dryness and the residue reconstituted in amino acid analysis buffer

Fig. 4.9 Characterisation of peptides PCS-1 and PCS-2 by HPLC

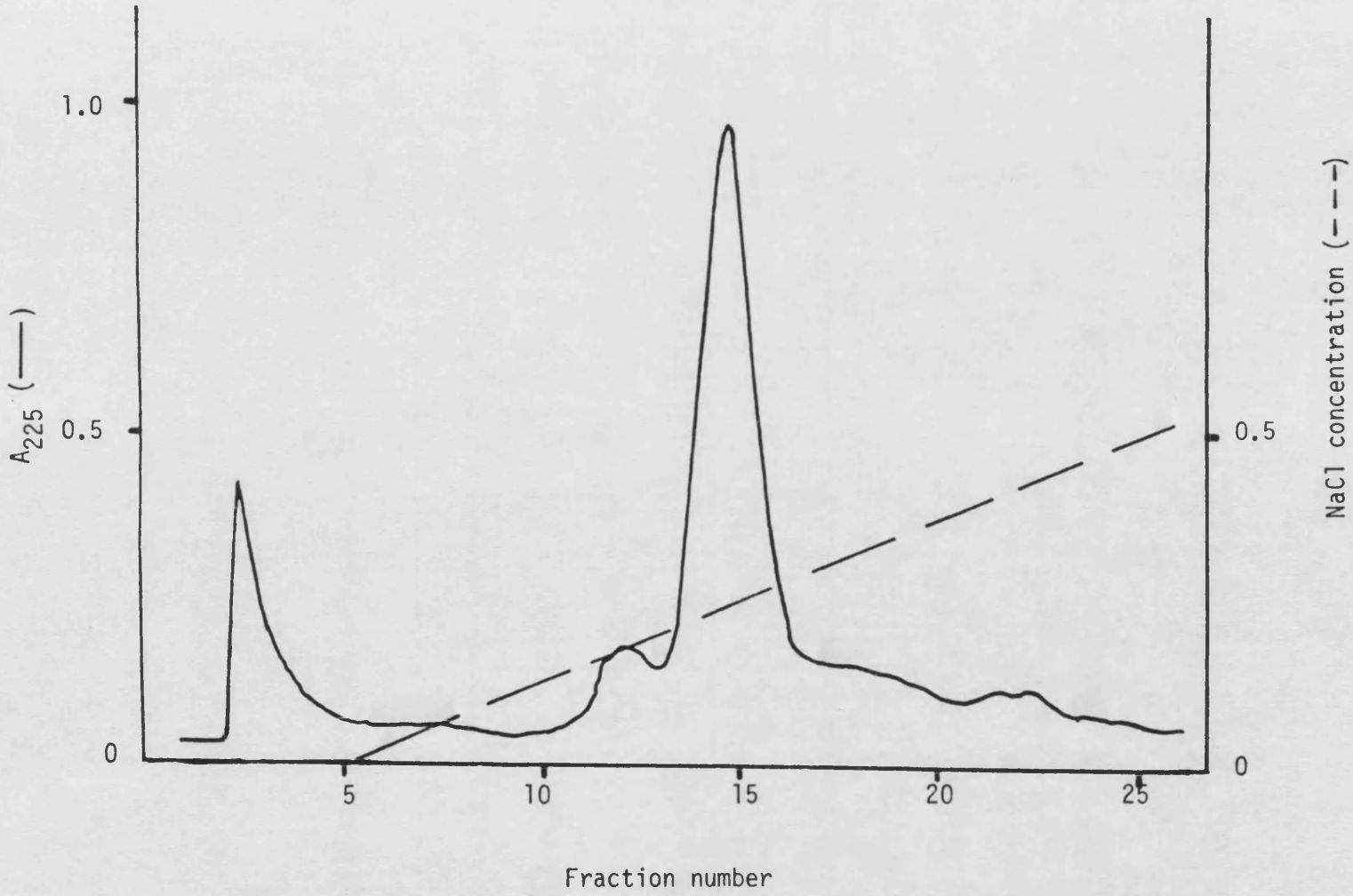


Peptides PCS-1 and PCS-2 analysed by HPLC as described in the text

Column C18 reverse phase

Gradient 5% - 95% acetonitrile in water

Fig. (4.10) Purification of PCS-1 peptide by cation-exchange chromatography (Mono-S) on FPLC



(25mM HCl) for analysis on an ion exchange amino acid analyzer using pH gradient elution. Samples were compared with standards for calculation of absolute levels of each amino acid. The results of analysis of samples of both peptides seem acceptable when compared with theoretical residues numbers. Table 4.4 shows the experimental results and theoretical composition for peptide PCS-1 at region 288-302, while Table 4.5 shows the results of peptide PCS-2 at region 76-90.

Table 4.4 Amino acid analysis of peptide PCS-1 at region (288-302)

residues	amino acid composition	
	experimental	theoretical
Arg	1.1	1
Asp	2.0	2
Gln	0.9	1
Glu	2.0	2
Gly	1.1	1
Leu	1.8	2
Lys	2.9	3
Ser	0.9	1
Val	1.9	2

Table 4.5 Amino acid analysis of peptide PCS-2 at region (76-90)

residues	amino acid composition	
	experimental	theoretical
Ala	1.0	1
Glu	3.2	3
Gly	1.9	2
Leu	1.8	2
Lys	2.9	3
Met	0.8	1
Pro	3.0	3

CHAPTER FIVE ANTIBODIES TO PHCS

5.1 INTRODUCTION

Polyclonal and monoclonal antibodies have been raised against intact PHCS in previous work in this laboratory (Brennand, 1987). All antisera were evaluated for their ability to react with intact PHCS and some of them with fragments of PHCS. Using a number of ELISA systems, the MAb's that were tested were all able to interact with intact PHCS to varying degrees.

5.2 REACTIVITY OF ANTISERA AND MAb's WITH INTACT PHCS

Reactivity of antisera and MAb's were tested against intact PHCS. Mouse anti-PHCS was used at dilutions in the range 1/20 - $1/10^5$ and MAb's were used at concentrations in the range 0.01-50 μg/ml.

The sandwich ELISA as described in section 3.7b, in which antigen was immobilized via polyclonal rabbit anti-PHCS, was used. The results are plotted as A_{405} versus dilution of antibody. Fig. 5.1 shows that the binding of mouse anti-PHCS sera to intact PHCS varies linearly with dilution in the range 1/20 - 1/250, and significant binding could be detected at antiserum dilutions up to 1/1000. Normal mouse serum binding was significantly less than the binding of mouse anti-PHCS sera in this region.

As shown in Fig. 5.2, the reactivity of MAb's B and C with intact PHCS resembled that of anti-PHCS serum (Fig. 5.1). There was no significant difference between the binding of MAb's B and C.

An identical pattern was obtained Fig. 5.3 when the direct coating assay used, as described in section 3.7a. Both MAb's (B, C) gave reactivities consistent with those obtained by the sandwich assay.

Fig. 5.1 Dilution curve of mouse anti-PHCS serum in sandwich ELISA

Substrate incubation was for 1 h.

Each point represents the mean of duplicate determinations.

(▲) mouse anti-PHCS serum, (□) normal mouse serum.

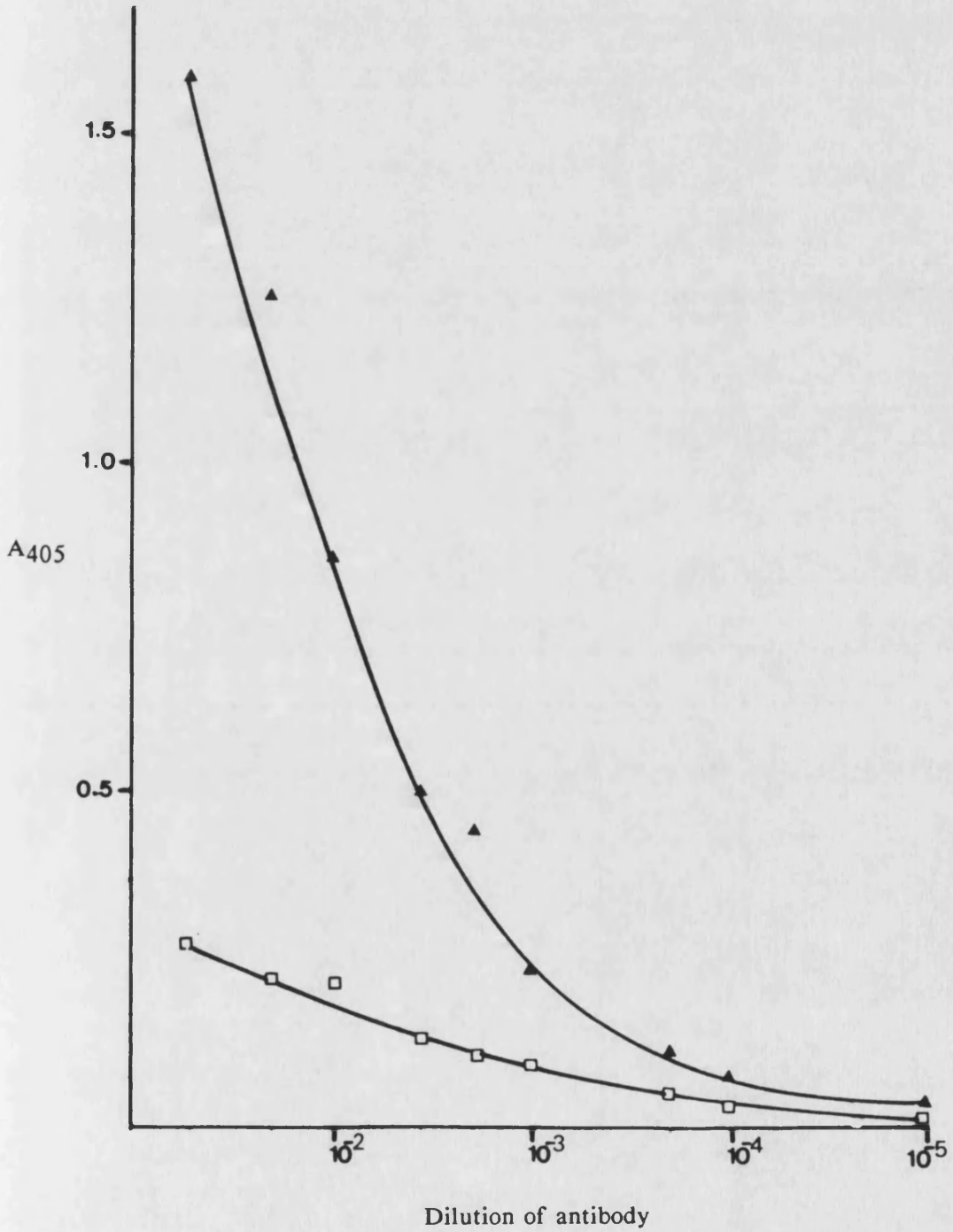


Fig. 5.2 Dilution curves for MAbs in sandwich ELISA

Substrate incubation was for 1h.

(■) MAb B, (●) MAb C, (□) normal mouse IgG.

Each point represents the mean of duplicate determinations.

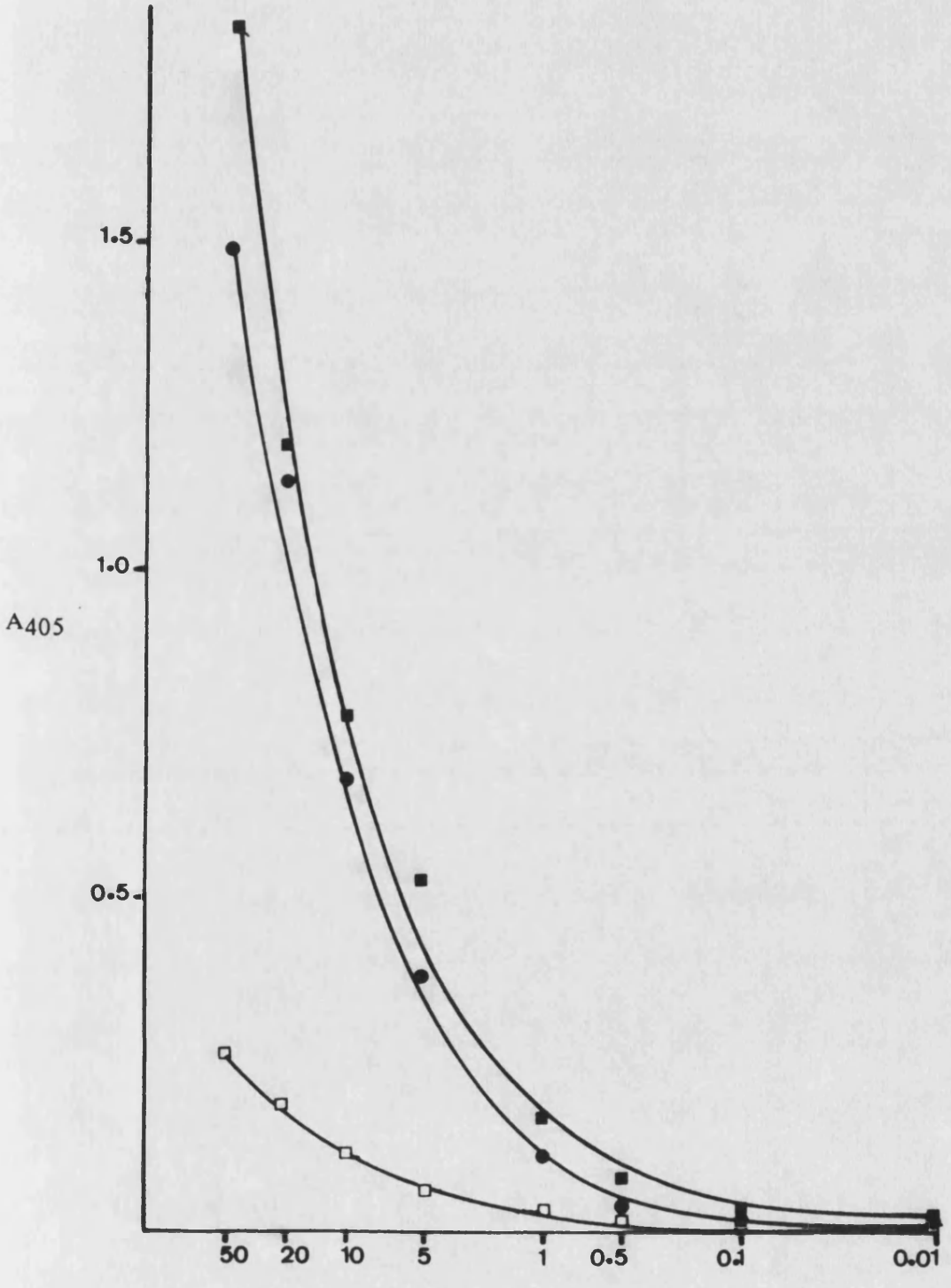
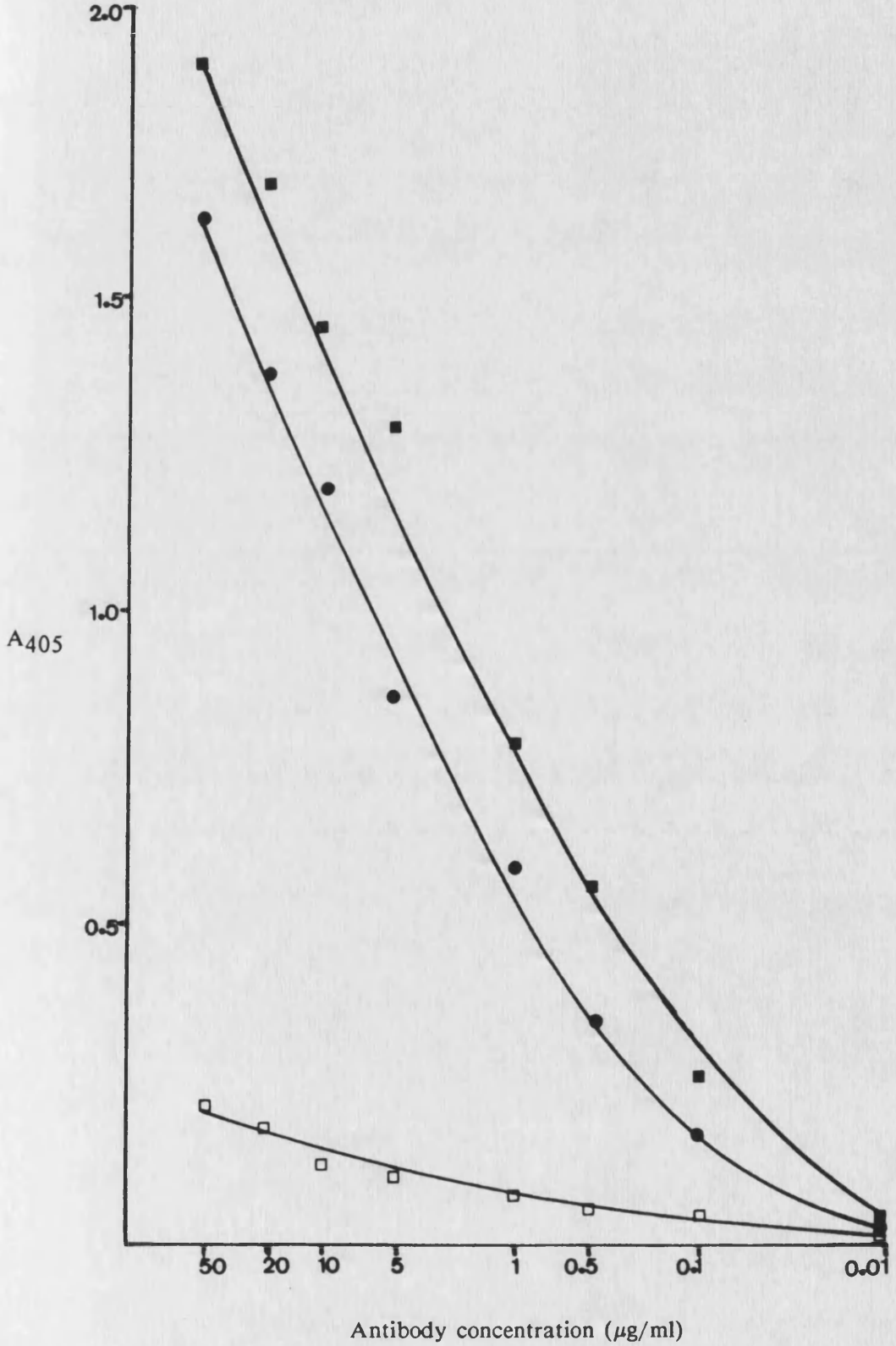


Fig. 5.3 Dilution curves for MAbs in direct ELISA

Substrate incubation was for 1h. (■) MAb B, (●) MAb C, (□) normal mouse IgG. Each point represents the mean of duplicate determinations.



5.3 INHIBITION OF ANTI PHCS SERA BY SOLUBLE PHCS

This assay was performed using the competitive ELISA as described in section 3.7c. A range of concentrations of PHCS (0-50 μg/ml) was used as competitor while the concentration of antibody used was fixed (1/100). Normal mouse serum was used as a control. Antibody activity in the presence of soluble PHCS was expressed as percentage of the activity measured in the absence of competitor. The relationship between the percentage of activity versus the competing antigen added is shown in Fig. 5.4; it can be seen that at a concentration of competing antigen of 50 μg/ml, the antibody binding was inhibited by 80%.

5.4 DIRECT ELISA USING SYNTHETIC PEPTIDE

The synthetic peptide PCS-1 at the range of concentrations 1, 2, 5, 10 μg/ml were used to determine the optimum plate coating concentration for the direct ELISA method. MAb E was used for optimization of plate coating, to define conditions for use with others MAb's. The plate was then exposed to a range of dilutions of MAb E, with normal mouse-IgG as control. The results obtained are shown in Fig. 5.5 as a graph of A_{405} versus the dilution of antibody. There was no significant difference between the dilution curves when different concentrations of peptide were used for coating. Therefore, a peptide concentration of 1 μg/ml has been chosen for use in all the following assays.

Fig. 5.4 Competitive sandwich ELISA

100% activity represents A_{405} = 1.55.

Substrate incubation was for 1h.

(▲) anti-PHCS, (□) normal mouse serum. Each point represents the mean of duplicate determinations.

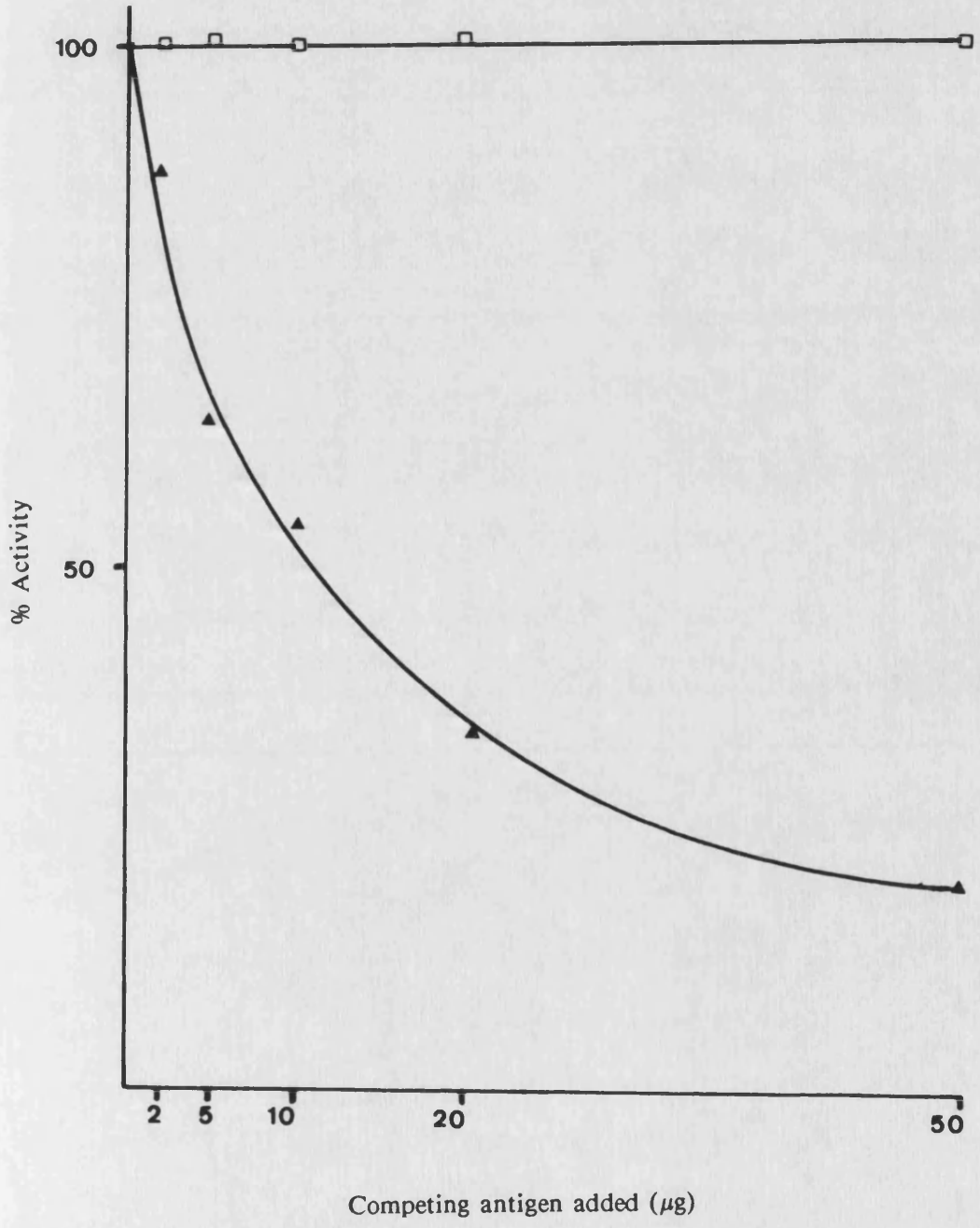
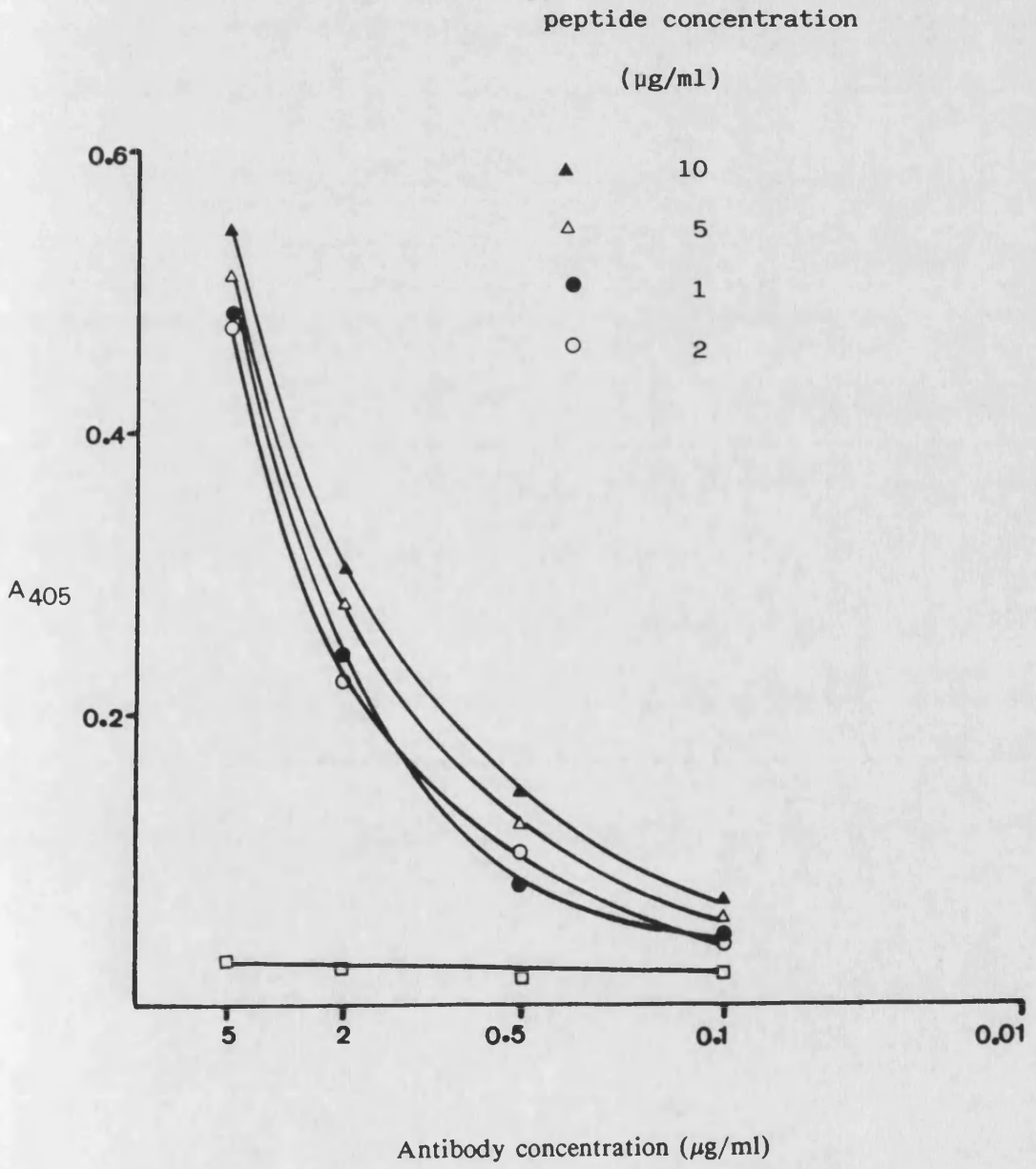


Fig. 5.5 Determination of optimum plate coating concentration for PCS-1 in direct ELISA

Substrate incubation was for 40min. (□) normal mouse serum.

Each point represents the mean of duplicate determinations.



5.5 COMPARISON BETWEEN REACTIVITIES OF POLYCLONAL AND MONOCLONAL ANTIBODIES WITH INTACT PHCS AND PEPTIDE

The reactivities of polyclonal anti-PHCS and MAb against PHCS and peptide PCS-1 were investigated using the direct ELISA method. The plates were coated with intact PHCS or peptide PCS-1 and were then exposed to a range of dilutions (0.5, 5, 50 μg/ml) of rabbit anti PHCS (polyclonal) and mouse-anti PHCS (MAb B). Normal rabbit-IgG and mouse IgG were used as controls. The results obtained are shown in Fig. 5.6 for polyclonal anti serum against intact PHCS and PCS-1, and in Fig. 5.7 for MAb B against both antigens. These results indicate that both MAb B and polyclonal anti-PHCS showed higher reactivity with intact PHCS than with peptide PCS-1.

5.6 REACTIVITY OF MAb's WITH SYNTHETIC PEPTIDES

As pointed out in section 5.1, in previous work by Brennand (1987) a library of 11 mouse monoclonal antibodies have been produced, using PHCS as immunogen. In the present work, the reactivity of each MAb with both synthetic peptides PCS-1 and PCS-2 has been examined using the direct ELISA method.

Peptides were immobilized on the solid phase for 18 h at 4°C, and then the plates were blocked with 1% (w/v) casein in PBS before exposure to MAb's. Absorbance values were plotted against antibody dilution. Fig. 5.8 shows the results when peptide PCS-1 was used as antigen, while Fig. 5.9 shows the results using PCS-2. The experiment was repeated twice. From these figures it can be seen that different MAb's show varying degrees of reactivity with each peptide. In the case of peptide PCS-1 (Fig. 5.8), the order of reactivity of MAb's is M>D>E>K>B>C>F>A>G>H>L, while with peptide PCS-2, (Fig. 5.9), the order of reactivity was slightly different (M>E>D>K>B>C>G>F>A>H>L). However, all reactions of MAb's with PCS-1 are greater than that with peptide PCS-2 except for MAbs E and G, as shown in Table 5.1.

Fig. 5.6 Comparison between reactivities of anti-PHCS with intact PHCS and PCS-1 in direct ELISA

Substrate incubation was for 30min.

(▲) anti-PHCS with CS, (●) anti-PHCS with PCS-1, (□) normal rabbit IgG with CS, (■) normal rabbit IgG with PCS-1. Each point represents the mean of duplicate determinations.

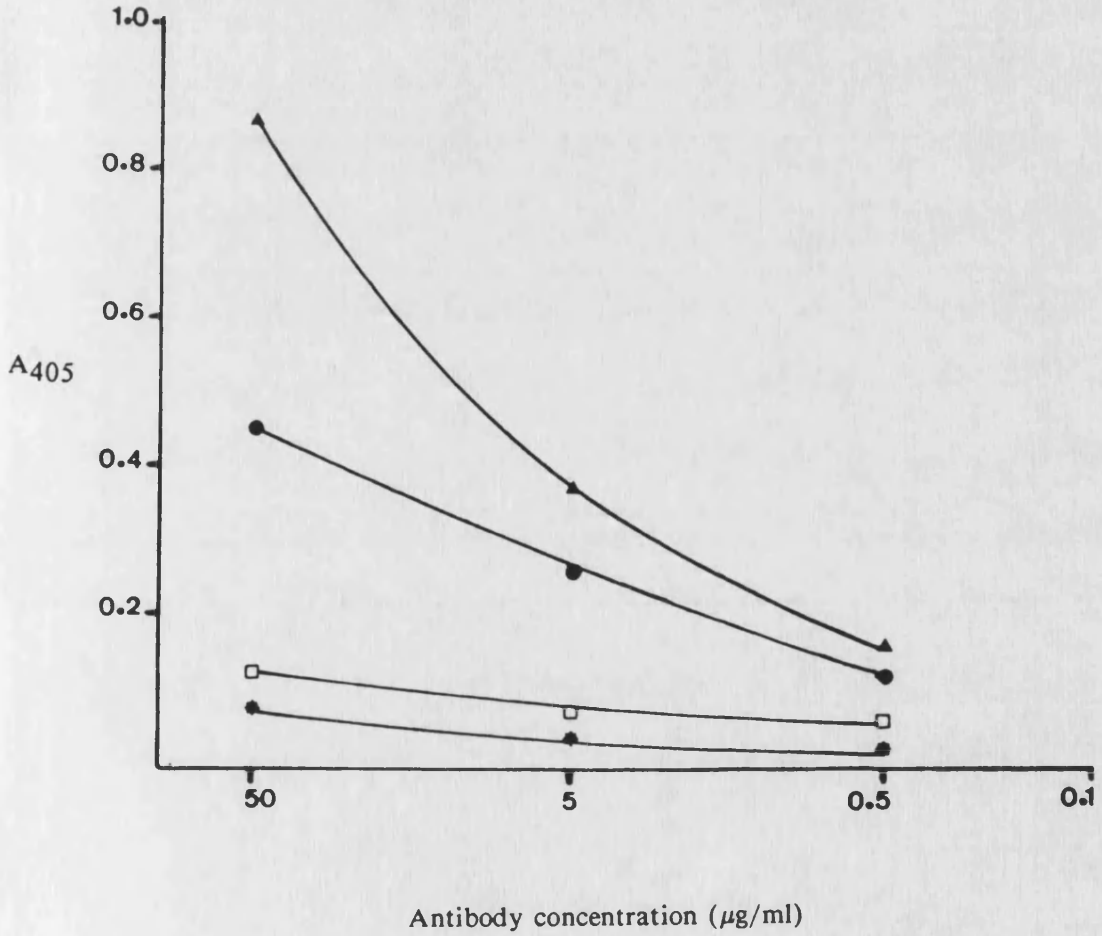


Fig. 5.7 Comparison between reactivities of MAb "B" with intact PHCS and PCS-1 in direct ELISA

Substrate incubation was for 70min.

(△) MAb with CS, (○) MAb with PCS-1, (□) normal mouse IgG with CS, (●) normal mouse IgG with PCS-1.

Each point represents the mean of duplicate determinations.

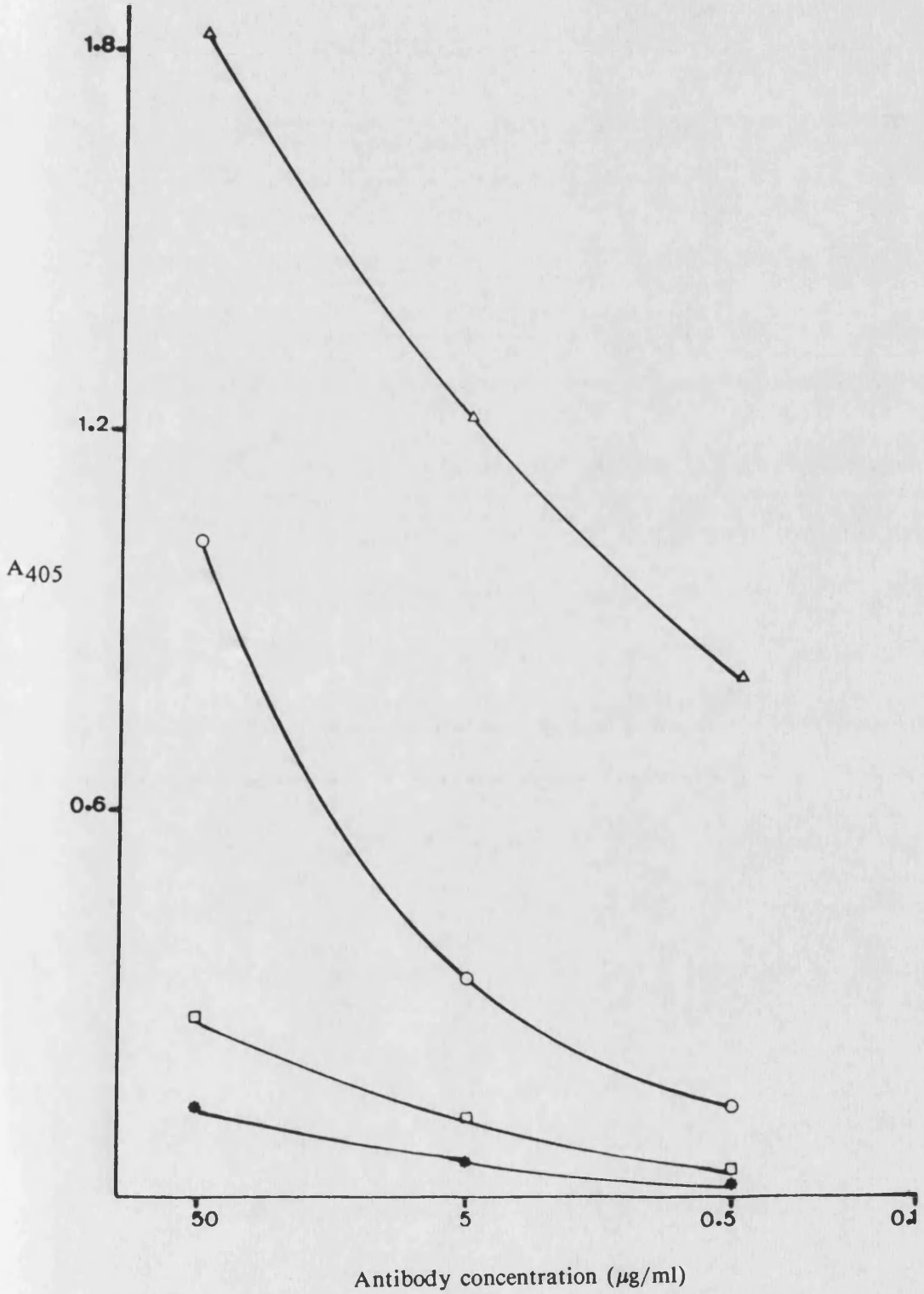


Fig. 5.8 Reactivity of MAbs with PCS-1 in direct ELISA

Substrate incubation was for 1h.

Results for MAbs M,D,E,K,B,C are indicated on the diagram. (- -) region represents the results for MAbs F,A,G,H,L. (□) represents normal mouse IgG.

Each point represents the mean of duplicate determinations.

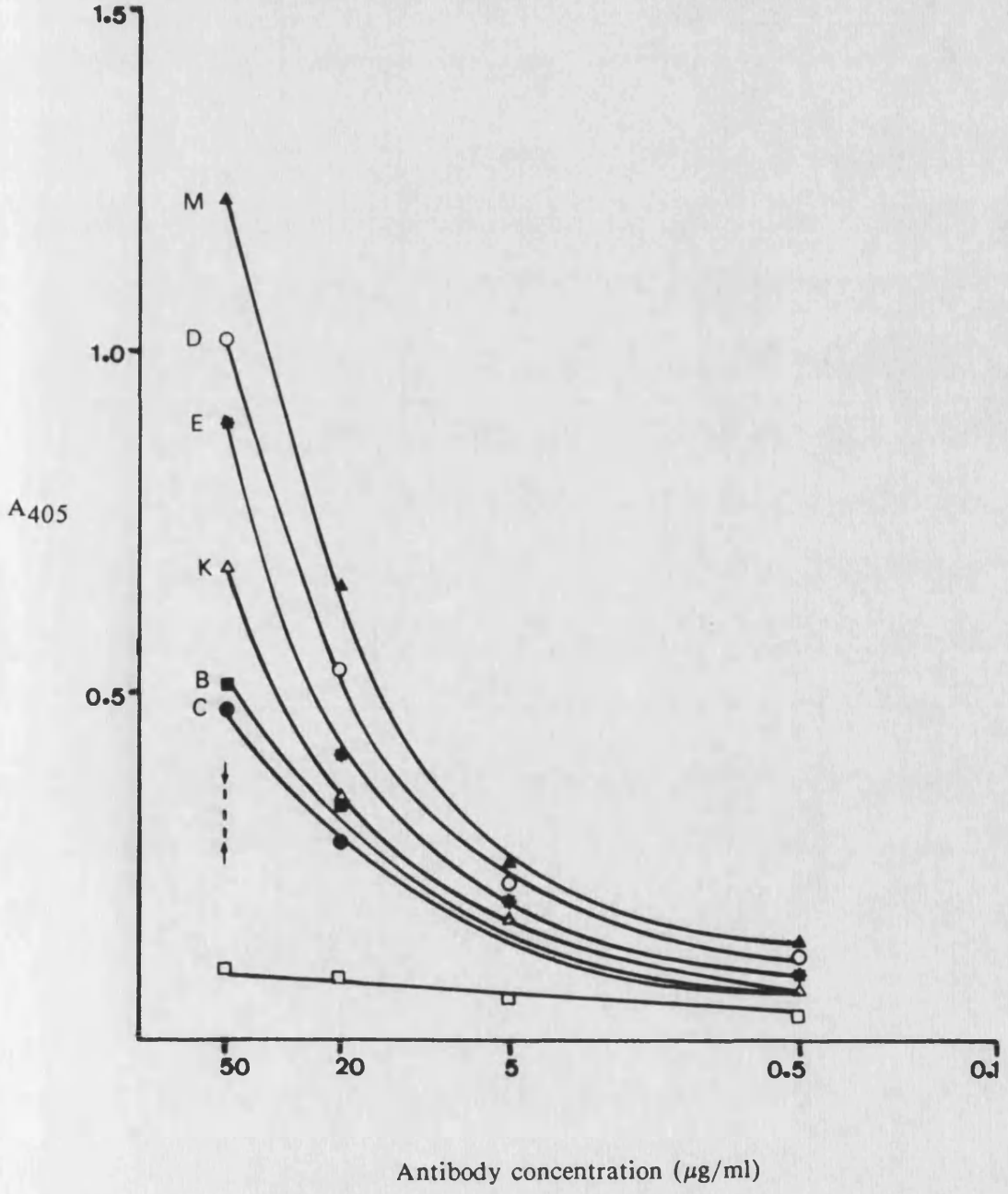


Fig. 5.9 Reactivity of MAbs with PCS-2 in direct ELISA

Substrate incubation was for 1h.

Results for MAbs M,E,D,K,B,C are indicated on the diagram.

(- -) region represents the results for MAbs G,F,A,H,L.

(□) normal mouse IgG. Each point is the mean of duplicate determinations.

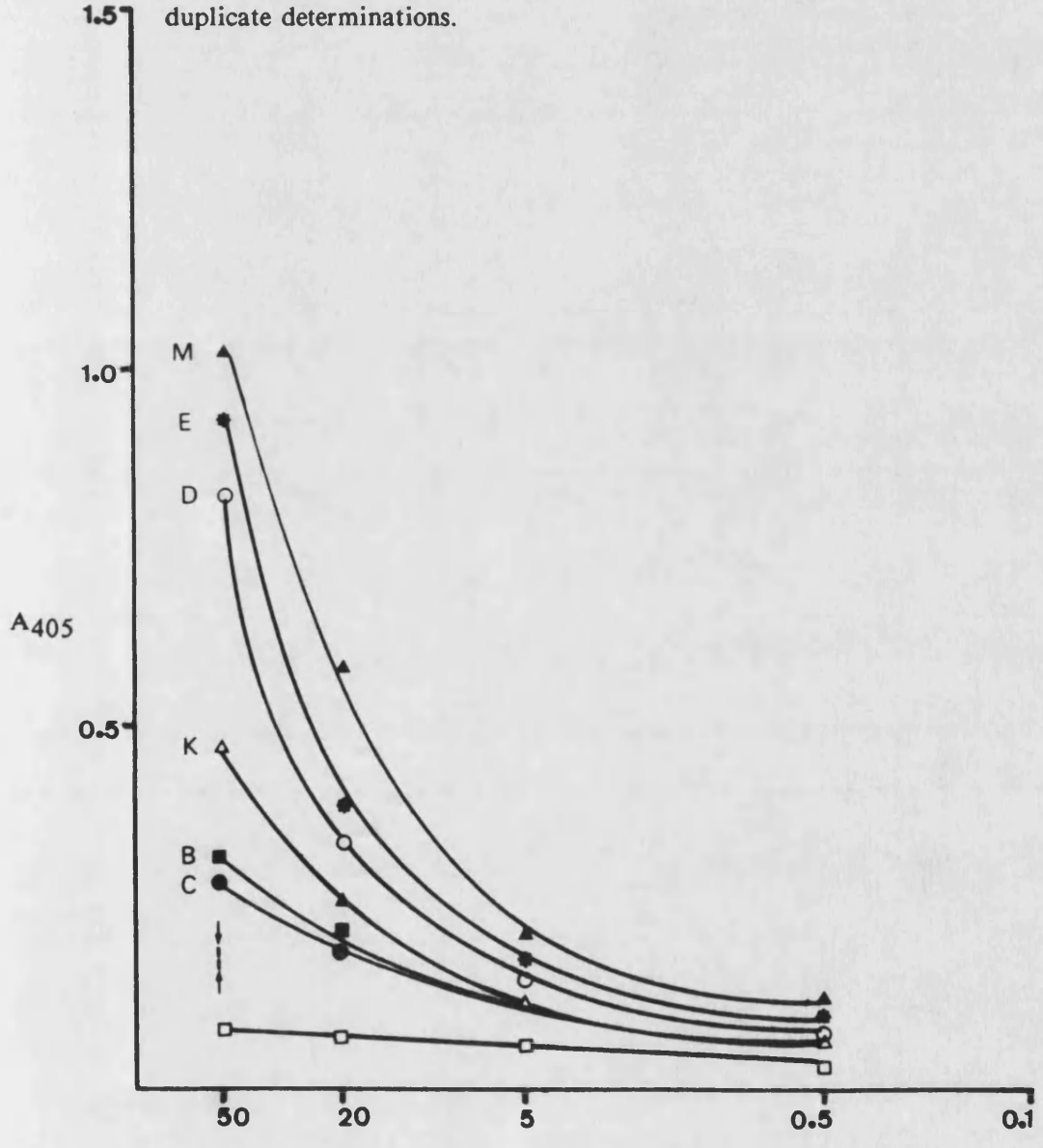


Table 5.1 Activity of MAb's with synthetic peptides

(results at 50 μg/ml MAb).

MAb	reactivity with	
	PCS-1	PCS-2
M	100%	100%
D	83.2%	78.8%
E	72%	90.3%
K	56%	46.1%
B	40.8%	30.7%
C	39.2%	27.8%
F	33.6%	24.2%
A	22.4%	21%
G	20%	26.4%
H	18.4%	16%
L	17.3%	13.2%

To examine the specificity of reaction of MAb's with peptides PCS-1 and PCS-2, a peptide (x-peptide) of 10 amino acid residues whose sequence is unrelated to the sequence of PHCS, has been chosen and used as a control peptide for the reaction. From the data explained above (Fig. 5.8 and 5.9), two different MAb's were selected (M and B) for use in this assay. The assay was carried out as described above, but it included a plate coated with 1 μg/ml of x-peptide as control. The results obtained are shown in Fig. 5.10. Both MAb's M and B showed reactivity with peptides PCS-1 and PCS-2 (similar to their reactivity as shown in Figs. 5.8 and 5.9) but also with x-peptide.

5.7 INHIBITION OF MAb B BY SOLUBLE PEPTIDE

Brennand (1987) found that MAb B could bind to intact PHCS and suggested that it bound to a site in the region 258-313. Since in this study, two different peptides have been synthesised chemically, corresponding to residues 288-302 for peptide PCS-1 and residues 76-90 for peptide PCS-2, inhibition of MAb "B" by both peptides was examined using competitive ELISA, as described in section 3.7c. In this assay, antibody was reacted with peptide in solution prior to assessment of antibody binding to intact PHCS on the solid phase. MAb B, at a fixed concentration (20 μg/ml), was incubated with varying concentrations of peptides (0.1, 1, 10, 100 μg/ml) for 1h at 37°C, and was then exposed to PHCS-coated plates. By plotting % inhibition of binding versus competing antigen (peptide) added, as shown in Fig. 5.11, it can be seen that peptide PCS-1 inhibited the reaction by up to 13%, while PCS-2 showed no effect.

Fig. 5.10 Reactivity of MAbs M and B with PCS-1, PCS-2 and x-peptide in direct ELISA

Substrate incubation was 75min.

(▲,△,●) MAb M with PCS-1, PCS-2, x-peptide respectively.

(●,○,•) MAb B with PCS-1, PCS-2, x-peptide respectively.

(□) normal mouse IgG. Each point represents the mean of duplicate determinations.

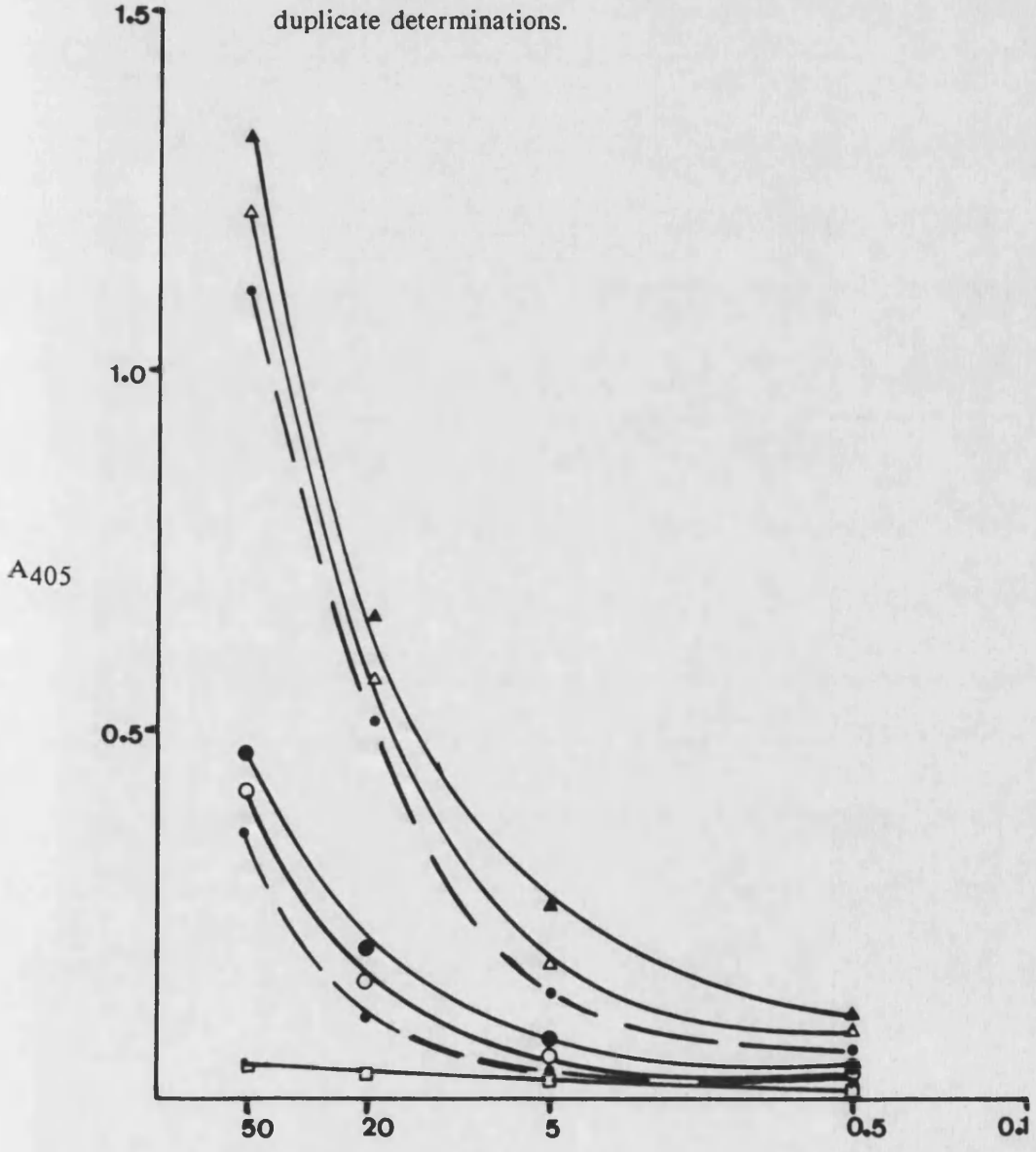
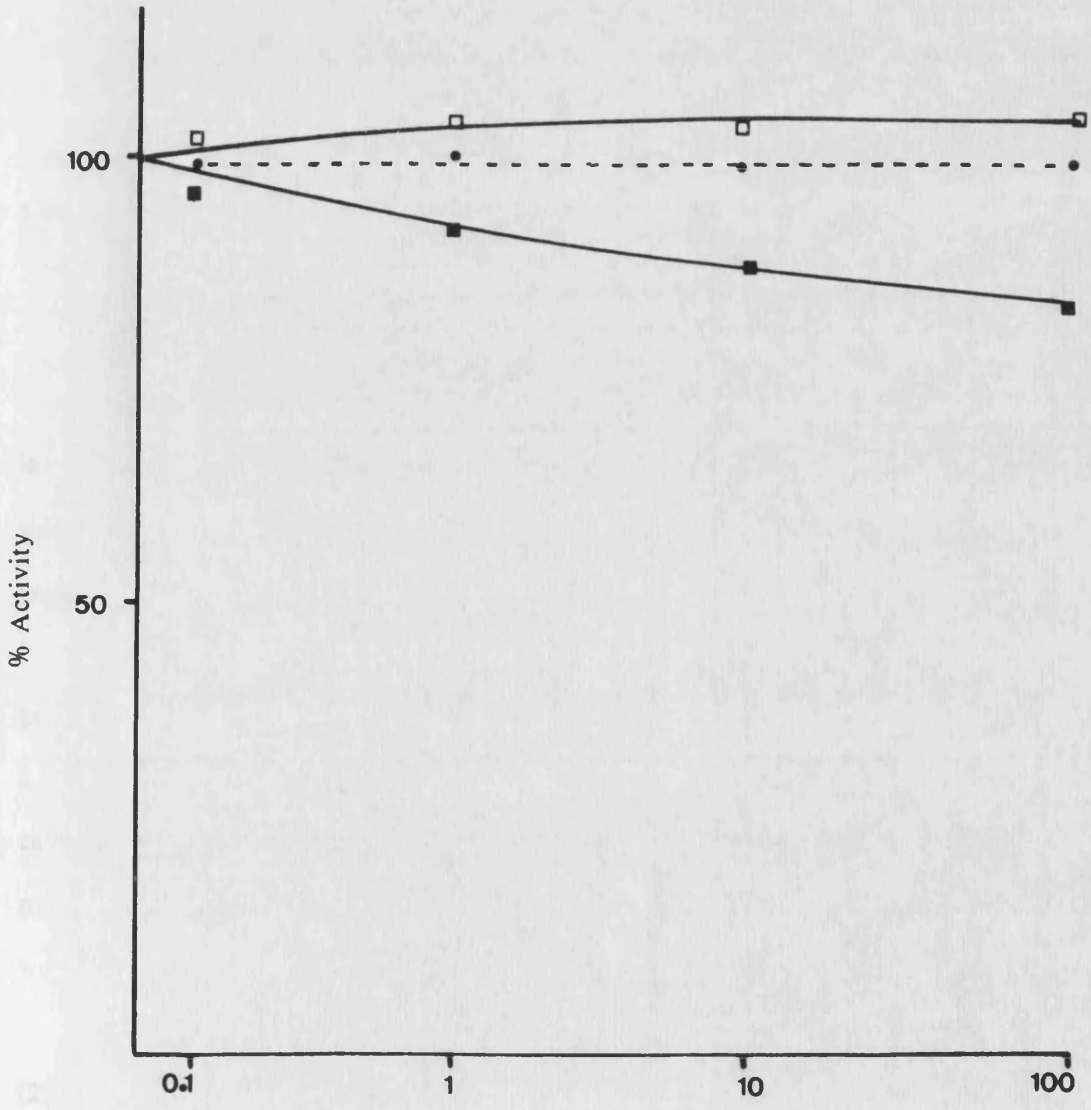


Fig. 5.11 Inhibition of MAb B by soluble PCS-1 and PCS-2 in competitive direct ELISA

Substratet incubation was for 45min.

(■) MAb B with PCS-1, (□) with PCS-2, (-•-) normal mouse IgG. Each point represents the mean of duplicate determinations.



5.8 INHIBITION OF MAb B BY SOLUBLE PHCS

In this assay, the competitive ELISA method was used as described in the previous section 5.7. MAb B, at a fixed concentration (10 μg/ml), was incubated with varying concentrations of competiting antigen (PHCS) 5, 15, 25, 50, 100 μg/ml for 1h at 37°C prior to exposure to a PHCS-coated plate.

The results obtained, plotted as the relationship between % activity and competiting antigen (PHCS) added, are shown in Fig. 5.12. It can be seen that PHCS inhibited the reaction of MAb B against PHCS by up to 16%.

5.9 FRAGMENTATION OF PHCS

a) by mild acid

PHCS was cleaved at asp-pro bonds by using mild acid (15 mM HCl), as described in section 3.8a. After hydrolysis for 90 min at 110°C, the products were separated by electrophoresis on an 8-25% (w/v) polyacrylamide gel, using the tris-bicine buffer system as described in section 3.11.

Complete hydrolysis yielded 5 peptides detectable on the stained gel (Fig. 5.13). A graph of Log_{10}%T against Log_{10} mol.wt. was plotted for the mol.wt. standards (Fig. 5.14). For each fragment produced following the cleavage, Log_{10}%T values were calculated from the distance travelled throughout the gel, and the corresponding mol.wts. were determined from the graph (Fig. 5.13). So, the mol.wts. of the peptides were: 4.9K, 8.7K, 13K, 17.5K and 21.3K.

Since the amino acid sequence of PHCS contains 4 asp-pro bonds [Fig. (2.2) page (27)] a complete and specific hydrolysis at asp-pro would yield 5 products containing residues 1-59, 60-257, 258-327, 328-344 and 345-437. The expected mol. wts. of the products of a limited hydrolysis were calculated, using the known mol. wts. of amino

Fig. 5.12 Inhibition of MAb by soluble PHCS in competitive direct ELISA

Substrate incubation was for 30min.

(■) MAb B, (-•-) normal mouse IgG. Each point represents the mean of duplicate determinations.

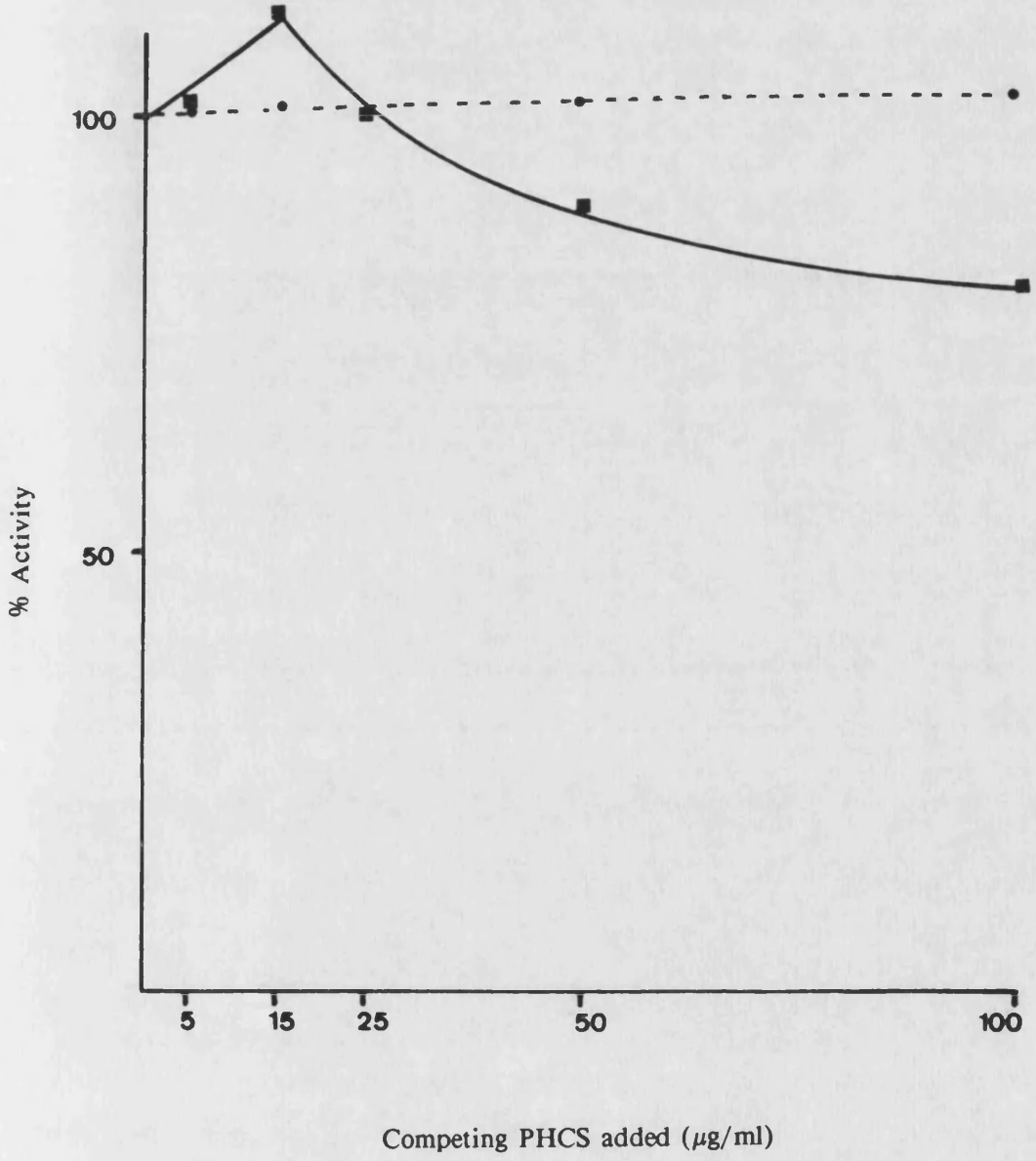
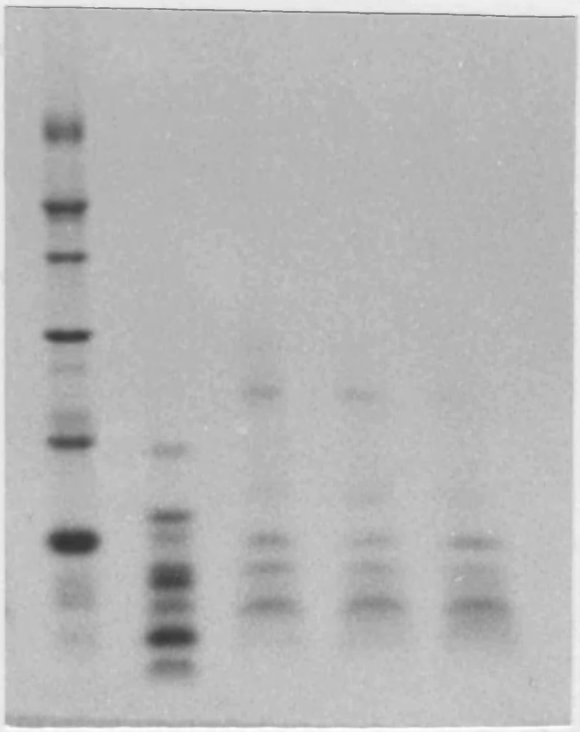


Fig. 5.13 Cleavage of PHCS at asp-pro bonds by dilute acid, shown on 8-25% SDS-PAGE



Track 1; Standards: 66k, 45K, 36K, 29K and 20K.

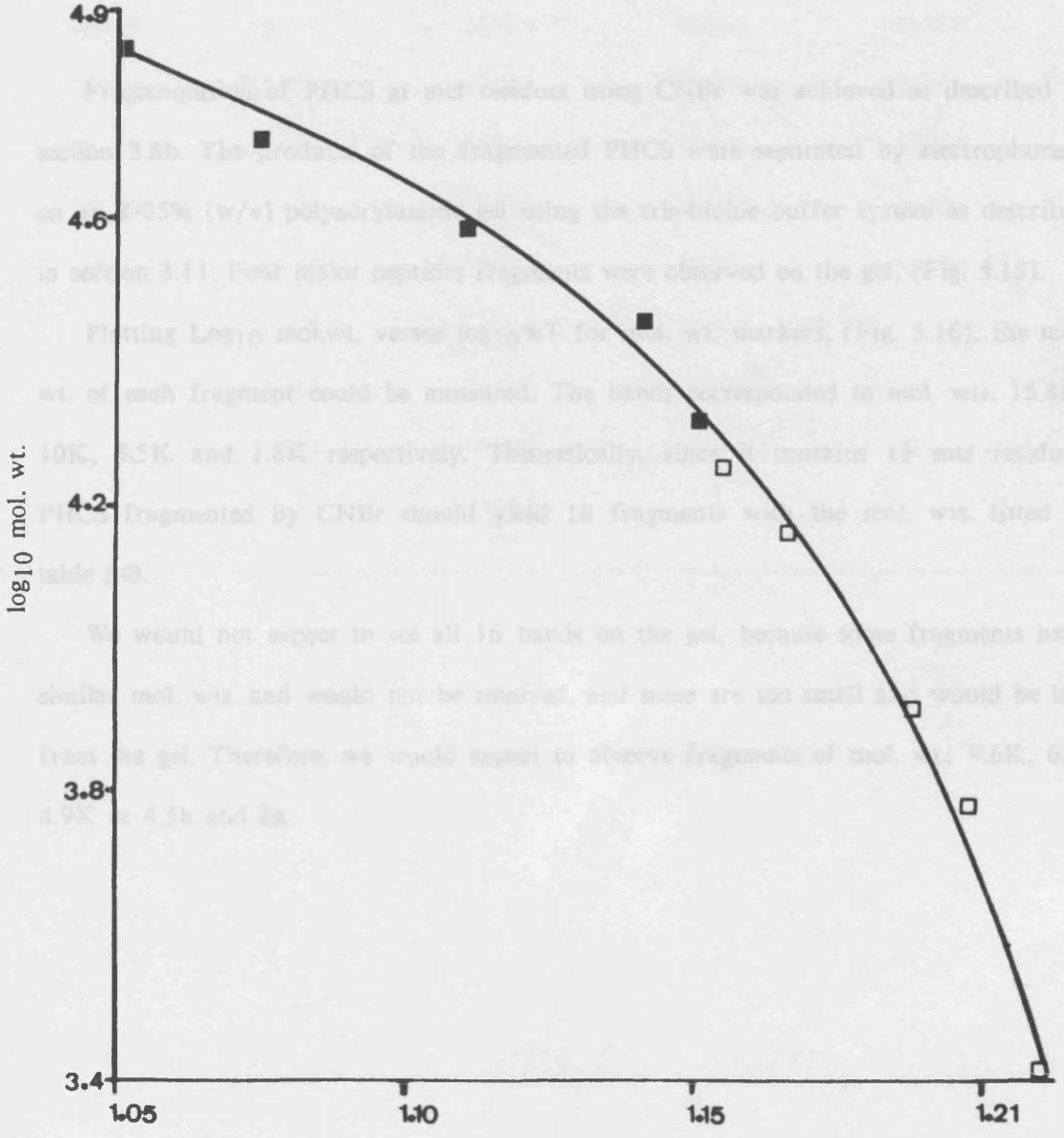
Track 2; Standards: 17K, 14.4K, 8.2K, 6.2K and 2.5K.

Track 3-5; mild acid digestion of PHCS.

Fig. 5.14 $\log_{10}$ mol. wt. versus log%T for the molecular weight standards shown in Fig. 5.13

(■) High mol. wt. standard, (□) Low mol. wt. standard.

Each point represents the mean of duplicate determinations.



acids, as shown in Table 5.2.

Comparison of the observed mol. wts. of fragments produced by mild acid cleavage with the predicted values shown in Table 5.2 suggests that complete cleavage of all asp-pro bonds was not obtained.

b) By CNBr

Fragmentation of PHCS at met residues using CNBr was achieved as described in section 3.8b. The products of the fragmented PHCS were separated by electrophoresis on an 8-25% (w/v) polyacrylamide gel using the tris-bicine buffer system as described in section 3.11. Four major peptides fragments were observed on the gel, (Fig. 5.15).

Plotting Log_{10} mol.wt. versus log_{10}%T for mol. wt. markers, (Fig. 5.16), the mol. wt. of each fragment could be measured. The bands corresponded to mol. wts. 15.8K, 10K, 5.5K and 1.8K respectively. Theoretically, since it contains 15 met residues, PHCS fragmented by CNBr should yield 16 fragments with the mol. wts. listed in table 5-3.

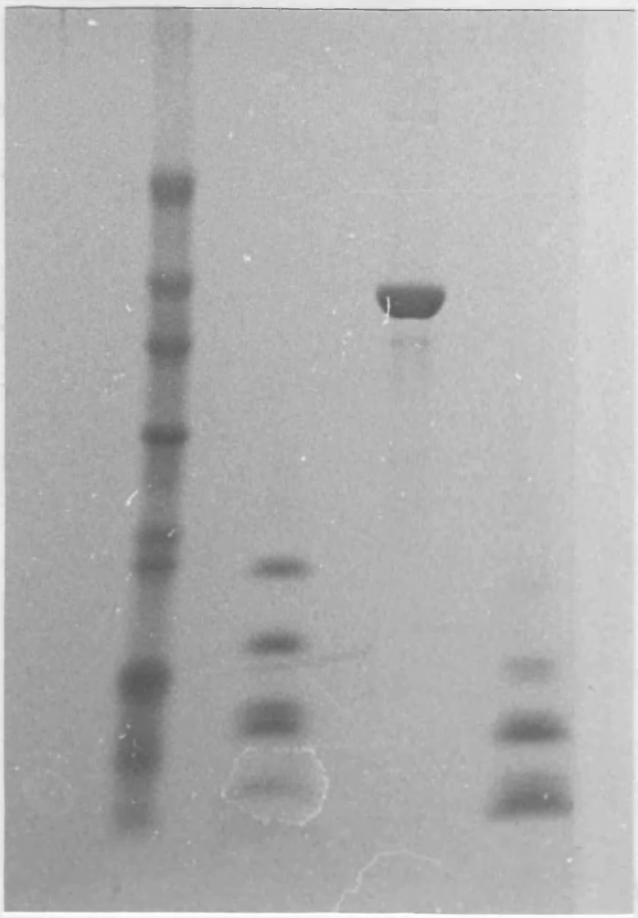
We would not expect to see all 16 bands on the gel, because some fragments have similar mol. wts. and would not be resolved, and some are too small and would be lost from the gel. Therefore, we would expect to observe fragments of mol. wt.: 9.6K, 6K, 4.9K or 4.5k and 2k.

Table 5.2 Theoretical products formed by cleavage of PHCS at asp-pro bonds

Molecular weights of fragments containing amino acid

1-59	60-257	258-327	328-344	345-437
48969	48969	48969	48969	48969
38500	42500	42500	42500	42500
36400	38500	38500	38500	20300
28700	36400	36400	32000	12600
6500	32000	32000	20300	10500
	29900	29900	12600	
	28700	20300	9800	
	22200	9800	2100	
		7700		

Fig. 5.15 Cleavage of PHCS at met residue by CNBr, shown on 8-25% SDS-PAGE



1 2 3 4

Track 1; Standards: 66k, 45K, 36K, 29K and 20K.

Track 2; Standards: 17K, 14.4K, 8.2K, 6.2K and 2.5K.

Track 3; undigested PHCS.

Track 4; CNBr-digested PHCS.

Fig. 5.16 $\log_{10}$ ___ mol. wt. versus log%T for the molecular weight standards shown in Fig. 5.15

(■) High mol. wt. standard, (□) Low mol. wt. standard.

Each point represents the mean of duplicate determinations.

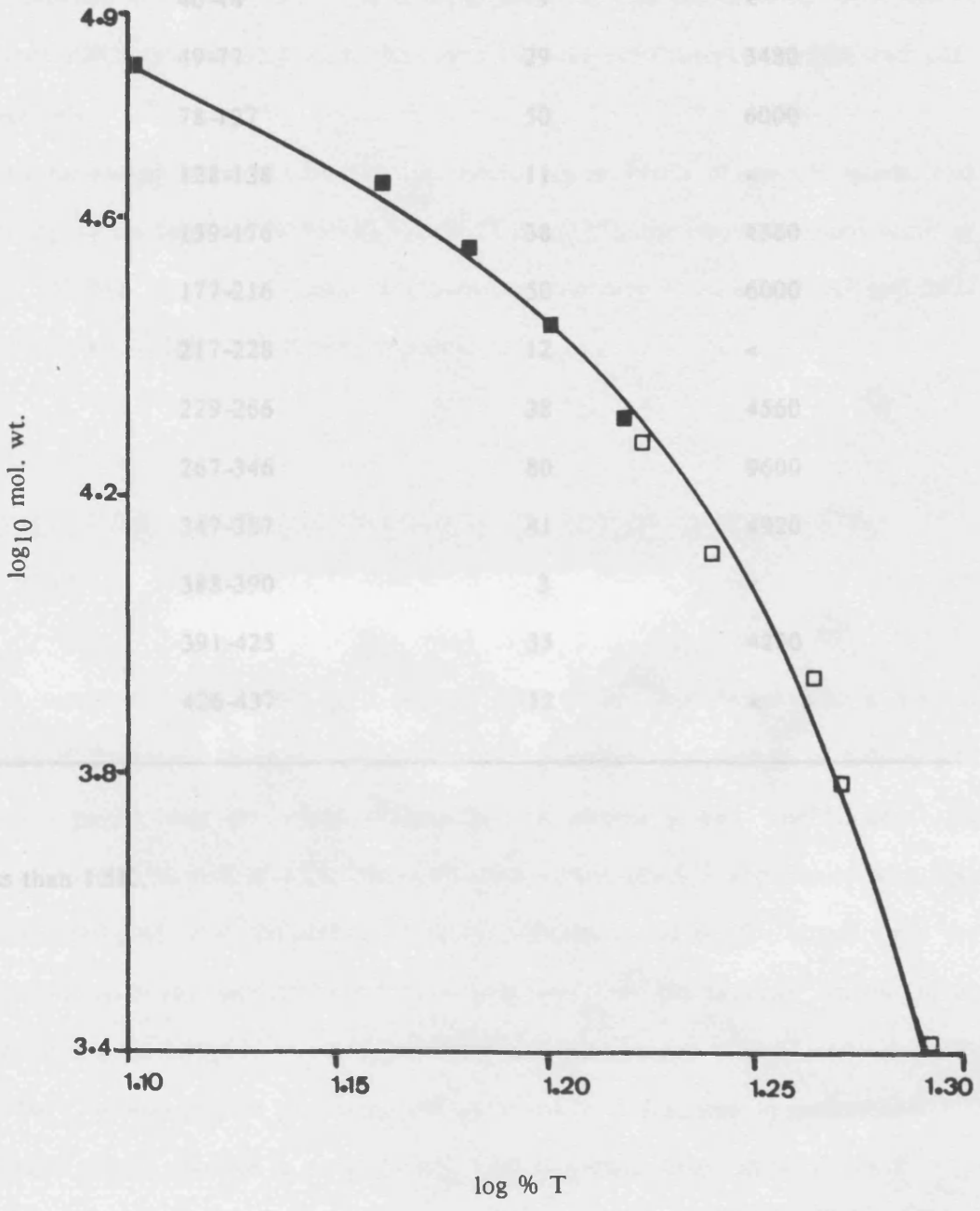


Table 5.3 Theoretical products formed by fragmentation of PHCS by CNBr

no.	frag.	size	M. Wt.
1	1-40	40	4800
2	41	1	<*
3	42-45	4	<
4	46-48	3	<
5	49-77	29	3480
6	78-127	50	6000
7	128-138	11	<
8	139-176	38	4560
9	177-216	50	6000
10	217-228	12	<
11	229-266	38	4560
12	267-346	80	9600
13	347-387	41	4920
14	388-390	3	<
15	391-425	35	4200
16	426-437	12	<

* less than 1.5K.

c) By hydroxylamine

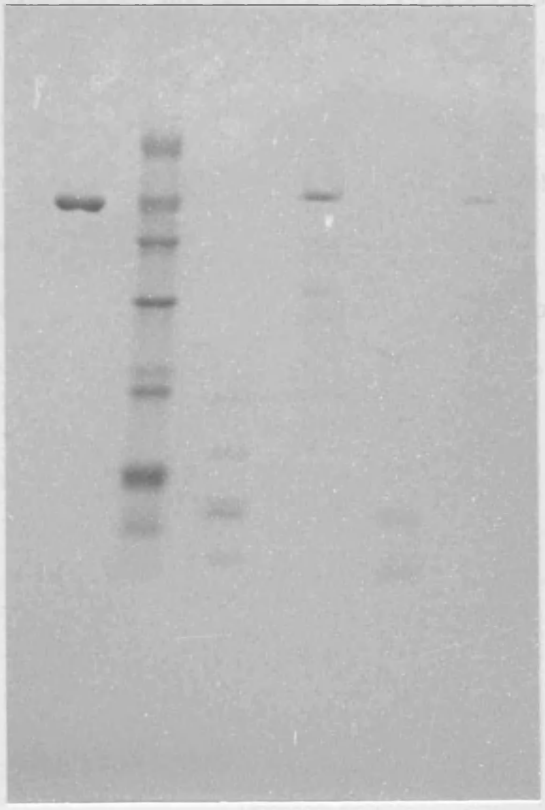
PHCS was cleaved at asn-gly bonds using hydroxylamine as described in section 3.8c. The products of the fragmented enzyme were separated by electrophoresis on an 8-25% (w/v) polyacrylamide gel, using the tris-bicine buffer system. The cleaved PHCS yielded 3 bands, although on the photograph of the gel (Fig. 5.17) only one major band can be observed, while the other two bands are not visible clearly. Determination of the mol. wt. of those bands indicated that one of them corresponded to intact PHCS (about 49K) while the others had molecular weights of 29K and 18K respectively.

As mentioned above, hydroxylamine should cleave PHCS at asn-gly bonds, and according to the sequence of PHCS, Fig. (2.2) page (27), the cleavage should occur at region 267-268 forming two major fragment corresponding to regions 1-267 and 268-437. (mol. wts. = 30K & 19K, respectively).

5-10 MAb IMMUNOSORBENT CHROMATOGRAPHY OF CNBr DIGESTED PHCS

A sample of PHCS (300 μg), cleaved with CNBr, was loaded onto a 1.5 ml column of Sepharose 4B-MAb B immunosorbent prepared as described in section 3.9. Chromatography was performed as described in section (3-10). The Column was washed with 15ml each of PBS, 20mM tris-HCl buffer, pH 8.0, and finally with 3ml of 0.5M NH_4OH. The NH_4OH wash should contain bound peptide eluted from the column as un-bound peptide should have been eluted in the previous washes. After freeze-drying the NH_4OH eluate, the product was reconstituted in H_2O and run on 8-25% (w/v) polyacrylamide gels, using tris-bicine buffer as described in section 3.11. The band pattern obtained is shown in Fig. 5.18. The bands observed in the track

Fig. 5.17 Cleavage of PHCS at asn-gly bonds by hydroxylamine, shown on 8-25% SDS-PAGE



1 2 3 4 5 6

Track 1; undigested PHCS.

Track 2; Standards: 66K, 45K, 36K, 29K and 20K.

Track 3,5; standards, 17K, 14.4K, 8.2K, 6.2K and 2.5K.

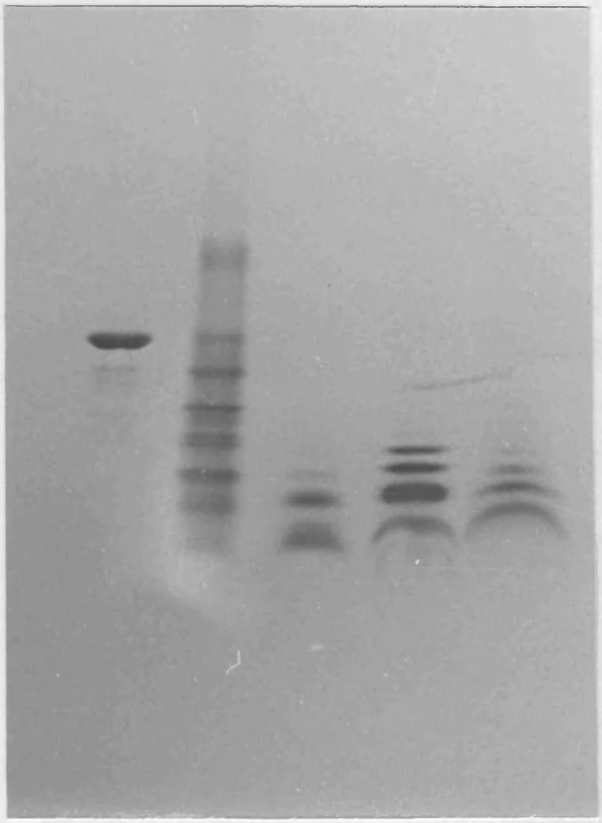
Track 4,6; hydroxylamine-digested PHCS.

loaded with the peptide eluted from the column with NH_4OH seem analogous to those bands appearing in the track loaded with a sample of fragmented PHCS.

According to these results, two assumptions can be made. It could be that the MAb is not specific for any one of these CNBr peptide fragments, or alternatively, it could be some protein was released from the column in this NH_4OH fraction.

These possibilities were tested by repeating the run and using a blank (PBS) instead of digested enzyme, to check if there was any MAb relased from the column. From the band pattern shown in Fig. 5.19, it can be seen that there is no band observed in track from the blank fraction, which means that there was no protein relased from the column by the NH_4OH wash. Therefore, although fragmentation of PHCS by using CNBr yielded fragments, no single fragment was specifically bound to MAb B-Sepharose.

Fig. 5.18 Affinity chromatography of CNBr-digested PHCS on MAb B column. Separation of products on 8-25% SDS-PAGE



Track 1; undigested PHCS.

Track 2; Standards: 66K, 45K, 36K, 29K and 20K.

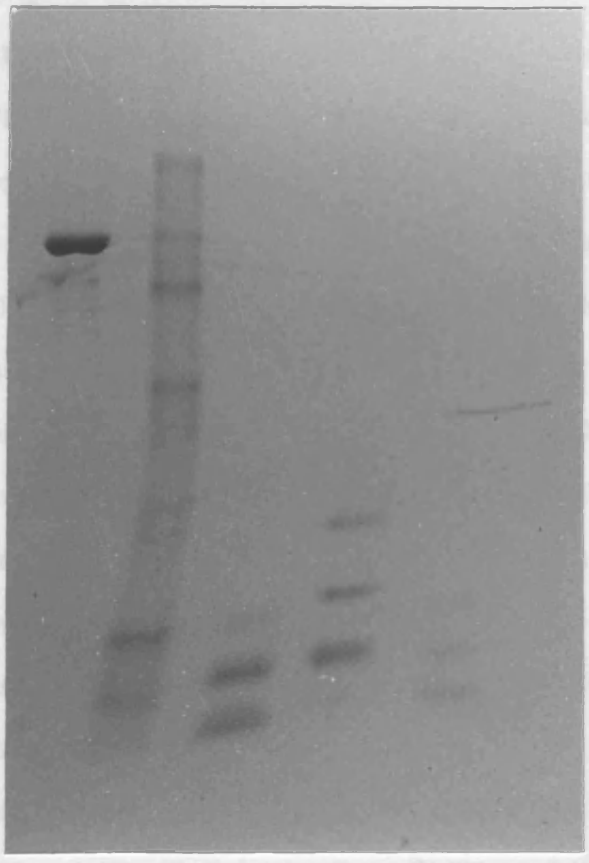
Track 3; CNBr-digested PHCS.

Track 4; Standards: 17K, 14.4K, 8.2K, 6.2K and 2.5K.

Track 5; material eluted in NH_4OH fraction.

Fig. 5.19 Affinity chromatography on MAb B column, blank control.

Separation of products on 8-25% SDS-PAGE



1 2 3 4 5 6

Track 1; undigested PHCS.

Track 2; Standards: 66k, 45K, 36K, 29K and 20K.

Track 3,5; CNBr-digested PHCS.

Track 4; Standards; 17K, 14.4K, 8.2K, 6.2K and 2.5K.

Track 6; Blank (PBS eluted in NH_4OH fraction).

5.11 DISCUSSION

A number of ELISA systems have been used to investigate the reactivity of polyclonal and MAb's raised against the enzyme PHCS. Intact PHCS and synthetic peptides corresponding to specific regions of the sequence of PHCS were used as antigens in these ELISAs.

In the sandwich ELISA technique, antigen is immobilized to polyclonal antibody-coated solid phase. The results obtained from this assay show that mouse anti-PHCS sera gave a strong cross-reactivity, particularly in the range of dilutions between 1/20 - 1/250 with intact PHCS, and significant binding could be evaluated at anti-sera dilutions up to 1/1000. On the other hand, normal mouse serum showed a very weak response compared with mouse-anti-PHCS serum.

Thus, both MAbs showed a significant interaction with PHCS in the sandwich ELISA, the immobilization method via the polyclonal antibody probably ensuring that the enzyme is presented in its native conformation. This interaction with native PHCS is consistent with our observations that the enzyme is effective in its soluble form in competitive ELISA assays, whereas in ELISA method where the PHCS-coating is direct with the plate, the enzyme maybe partially denatured.

Synthetic peptide PCS-1 has been used as the immobilized antigen at various concentrations (1, 2, 5 and 10 μg/ml) in the direct ELISA to determine the optimum concentration which should be applicable for coating. For this purpose, MAb E was used to define the conditions with other MAbs. The results of this assay indicate that there is no significant variation between the interaction of MAb E with the different peptide coated-concentrations. Therefore, for reasons of economy, peptide at 1 μg/ml was chosen for use in the following assays.

As mentioned earlier in section 5.1, a library of 11 monoclonal antibodies has been previously produced against intact PHCS. All of these MAb's were evaluated for their ability to interact with the synthetic peptides PCS-1 and PCS-2 in this study, using

the direct ELISA. The results obtained from this assay showed that the MAb's displayed varying degrees of interaction with both peptides. In the case of peptide PCS-1, the order of reactivity of these MAb's is: M>D>E>K>B>C>F>A>G>H>L. In the case of PCS-2 the order of reactivity slightly was different from that with PCS-1. MAb M gave the highest reactivity with both peptides, so, its value at 50 μg/ml was taken as 100% activity and the relative percentage activities of all others MAb were calculated with respect to it, (Table 5.1). From that table, it can be seen that most MAb's show a higher reactivity with peptide PCS-1 than with PCS-2, although some of them show a weak reactivity with both peptides.

The MAb's thus reacted with both peptides, although these peptides are at different regions of PHCS molecules as pointed out in section 4.3. Therefore, to investigate the specificity of reaction of the MAb's with these peptides, a random peptide (x-peptide) of 10 amino acid residues (cys-met-val-glu-arg-tyr-val-arg-pro-cys) was selected for use as a control for the ELISA reaction. Two MAb's were used for the assay, MAb M (which showed the highest reactivity with both peptides) and MAb B. It appears from the results obtained that MAb's M and B reacted with both peptides (PCS-1 and PCS-2) as expected from the previous data, but they also reacted with x-peptide, albeit to a lesser degree. This suggests that some of the observed MAb-binding to immobilized peptides may be an artifact of the assay system.

The direct ELISA method has also been used, to compare the reactions of polyclonal and monoclonal antibodies with intact PHCS and synthetic peptide PCS-1. MAb B and rabbit anti-PHCS were used and it was found, for both antibodies, the reaction with intact PHCS was stronger than with peptide.

The binding of anti-PHCS serum to intact PHCS was investigated using the competitive ELISA method. The native PHCS was used as a competitor at varying concentrations, and was incubated with a fixed dilution of anti-PHCS sera prior to exposure to the antigen (PHCS)-coated solid phase. It was found that the binding of anti-PHCS sera to immobilized PHCS is inhibited by 80% by soluble PHCS. Using the

same ELISA technique, MAb B was inhibited by soluble intact PHCS by approximately 16%.

The binding of MAb B to immobilized PHCS has also been examined for inhibition by soluble synthetic peptides PCS-1 and PCS-2 using the competitive ELISA method. It was found that MAb B is slightly inhibited by PCS-1 (up to 13% inhibition), while PCS-2 had no effect on the MAb binding.

The enzyme PHCS was chemically fragmented at specific sites by a variety of reagents: mild acid (15 mM HCl), hydroxylamine and CNBr.

In the case of mild acid, PHCS should be selectively cleaved at asp-pro bonds. After incubation of the PHCS for 90 min in mild acid, five major fragments were found of mol. wts. : 4.9K, 8.7K, 13K, 17.5K and 21.3K.

PHCS has 4 asp-pro bonds in its sequence and therefore complete cleavage should give 5 fragments with molecular weights; 2.1K, 6.5K, 7.7K, 10.5K and 22.2K.

From the data above, it can be concluded that complete cleavage at asp-pro bonds was not achieved, as shown by comparison between the mol.wt. values of the two sets of fragments (experimentally produced and predicted).

Hydroxylamine cleaves at asn-gly bonds. Treatment of PHCS produced 3 fragments corresponding to the native enzyme with mol.wt. approximately 49K and the two peptides of mol.wt. 29K and 18K.

In the sequence of the PHCS, there is only one asn-gly bond (267-268). Therefore, theoretically two major fragment should formed one at region 1-267 of mol.wt.~30K and the other at region 268-437 of mol.wt.~19K. The results obtained from the data above indicate that the mol.wt. of fragments produced experimentally using hydroxylamine corresponded to those predicted from the sequence.

CNBr cleaves at met residues. After digestion of PHCS by CNBr, 4 major fragments were produced with mol.wts. : 15.8K, 10K, 5.5K and 1.8K respectively.

From Table 5.3 it can be seen, that according to the amino acid sequence of PHCS molecule of 437 residues, there are 15 met residues exists. Therefore, it would be

expected to yield 16 fragments when digested by CNBr as listed in the table above. Not all these fragments could be observed when the digested enzyme was separated by gel electrophoresis, due to the fact that some of them are very small and some have similar mol.wts. The major bands which could be detected were 9.6K, 6K, 4.5K and 2K.

Since the fragments of PHCS produced by mild acid and hydroxylamine were studied in previous work for their binding to MAb's B and C by using immunoblot technique. Therefore, in this study the CNBr-fragments of PHCS have been examined for their ability to bind MAb B by using the immunosorbent chromatography technique. More information will be presented in the next chapter (section 6.9), about the immunobloting of these fragments with antisera to synthetic peptides and anti-PHCS serum. However, a MAb B immunosorbent column was used and from the results obtained, it can be seen that there is no significant difference between the band pattern detected in the track loaded with the peptide eluted from the column with NH_4OH and those bands observed in the track loaded with fragmented PHCS. This means, that MAb B might not be specific for any one of these fragments, thus no single peptide was retained specifically in the column by binding to the MAb. An alternative possibility that protein was released from the column was studied by using a blank (PBS) instead of the digested PHCS sample. The failure to detect any band in the track loaded from the blank fraction indicates that there was no protein released from the column by the NH_4OH wash. So, it can be concluded that no single fragment was specifically bound to MAb B-Sepharose.

CHAPTER SIX ANTIBODIES TO SYNTHETIC PEPTIDES

6.1 INTRODUCTION

Polyclonal antibodies have been raised to synthetic peptides PCS-1 and PCS-2, which correspond to regions of the PHCS molecule between residues 288-302 and 76-90, respectively. Rabbits were immunized with these peptides after conjugation to a carrier (KLH) as described in section 3.6. Then antibodies produced were detected in serum samples using ELISA techniques.

6.2 DETECTION OF ANTIBODIES IN ANTI-PEPTIDE SERA

As described in section 3.6, for each immunizing peptide two rabbits were used and bled after each boosting injection. Five samples were taken from each animal in total. The direct ELISA was used to detect and compare the antibody levels in serum samples. EIA plates were coated with peptide PCS-1 or PCS-2 (1 μg/ml) and the assays were carried out as described in section 3.7a. Plotting the absorbance at 405 nm versus serum dilution, the results obtained are shown in Fig. 6.1 and Fig. 6.2.

It can be seen from Fig. 6.1 and Fig. 6.2, with an increased number of booster injections, the level of serum antibody against peptide antigen increased and reached its highest level in bleed number five. The patterns were similar for the antisera from both rabbits immunized with the same immunogen. Therefore, antisera from the fifth bleed were used in all the following assays.

Fig. 6.1 Dilution curves of anti-PCS-1 sera using direct ELISA. Results from bleeds 1-5 are indicated on the figures

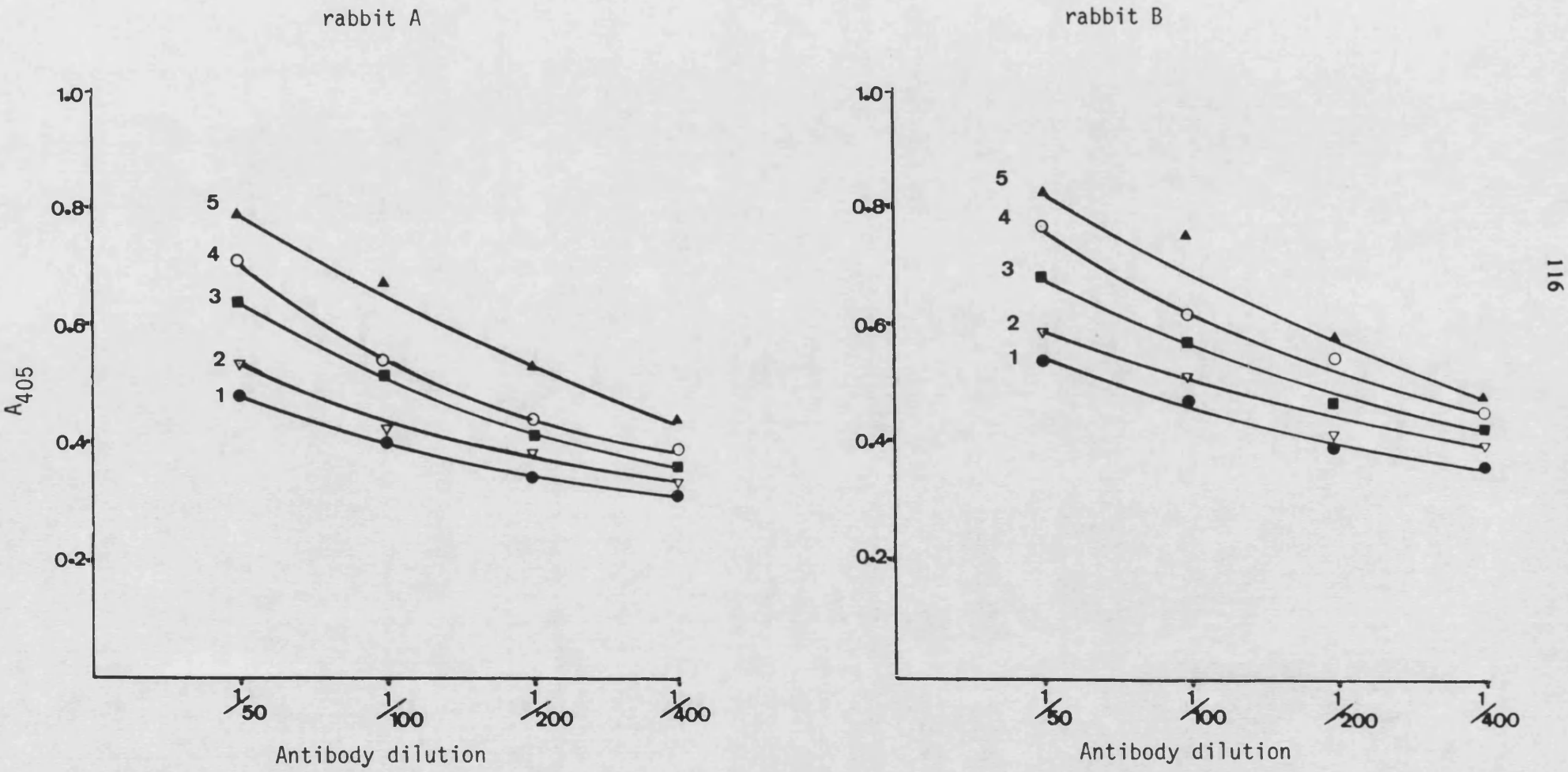
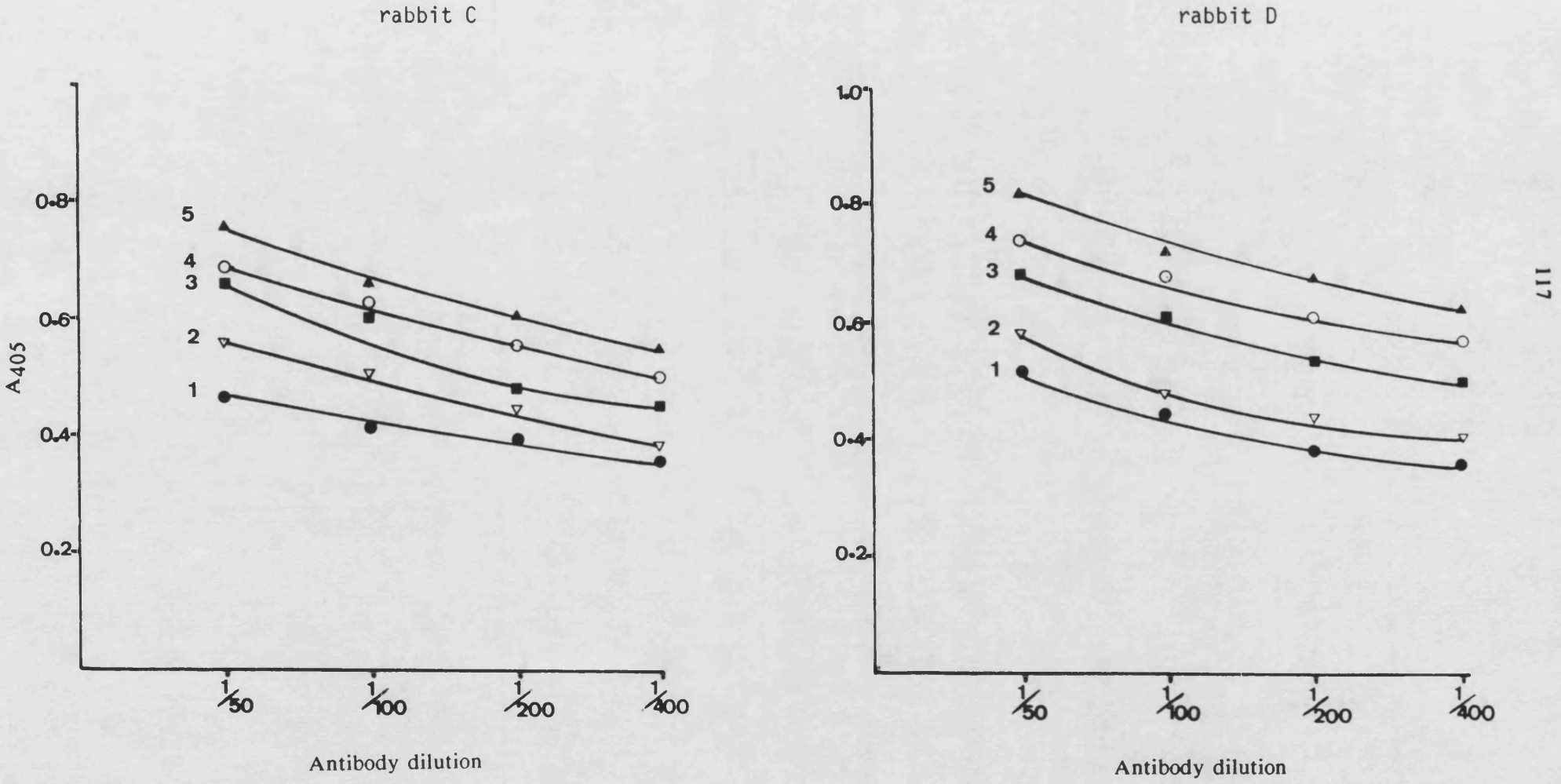


Fig. 6.2 Dilution curves of anti-PCS-2 sera using direct ELISA
Results from bleeds 1–5 are indicated on the figures



6.3 SPECIFICITY OF ANTI-PEPTIDE SERA

The specificity of anti-peptide sera was determined by using the ELISA technique. Samples of both synthetic peptides PCS-1 and PCS-2 were used as antigens in the direct ELISA, and these were incubated with a range of dilutions of anti-peptide serum, anti-KLH (carrier) sera, or normal rabbit serum as control. Following incubation with antibody enzyme-conjugate (alkaline phosphotase), substrate was added and the resulting absorbance at 405 nm measured.

Plots of A_{405} against serum dilution are shown in Fig. 6.3 and Fig. 6.4 for peptide PCS-1 and PCS-2, respectively. Fig. 6.3 indicates that only antibodies raised against peptide PCS-1 reacted with peptide PCS-1, while the others antibodies, raised against peptide PCS-2 and carrier (KLH), did not show reactivity with the PCS-1-coated plate. Furthermore, normal rabbit serum, which was used as a control, gave no reaction.

Fig. 6.4 shows the reactivity of anti-PCS-2 with peptide PCS-2-coated plate, while the other antibodies (anti-PCS-1, anti-KLH and normal rabbit serum) did not react with peptide PCS-2. From Fig. 6.3 and Fig. 6.4 it can be seen that antibodies from both rabbits which were immunized with the same immunogen showed similar reactivity.

Both antibodies (anti-peptide PCS-1 and anti-peptide PCS-2) gave a positive reaction, when incubated with the intact enzyme PHCS, as shown in Fig. 6-5. In this case, PHCS was used as an antigen, and was immobilized on the plate before exposure to the antibodies. The pattern shown in Fig. 6.5 indicates that anti-PHCS gave a higher reactivity with PHCS than anti PCS-1 or anti PCS-2, although anti PCS-1 showed higher reactivity than anti-PCS-2. The pattern was similar for both rabbits immunized with the same immunogen.

Fig. 6.3 Specificity of anti-peptide sera in direct ELISA against PCS-1

Substrate incubattion was for 45min.

(●, ○) anti-PCS-1 in both rabbits, (■) anti PCS-2, (▼) anti- KLH, (□) normal rabbit serum. Each point represents the mean of duplicate determinations.

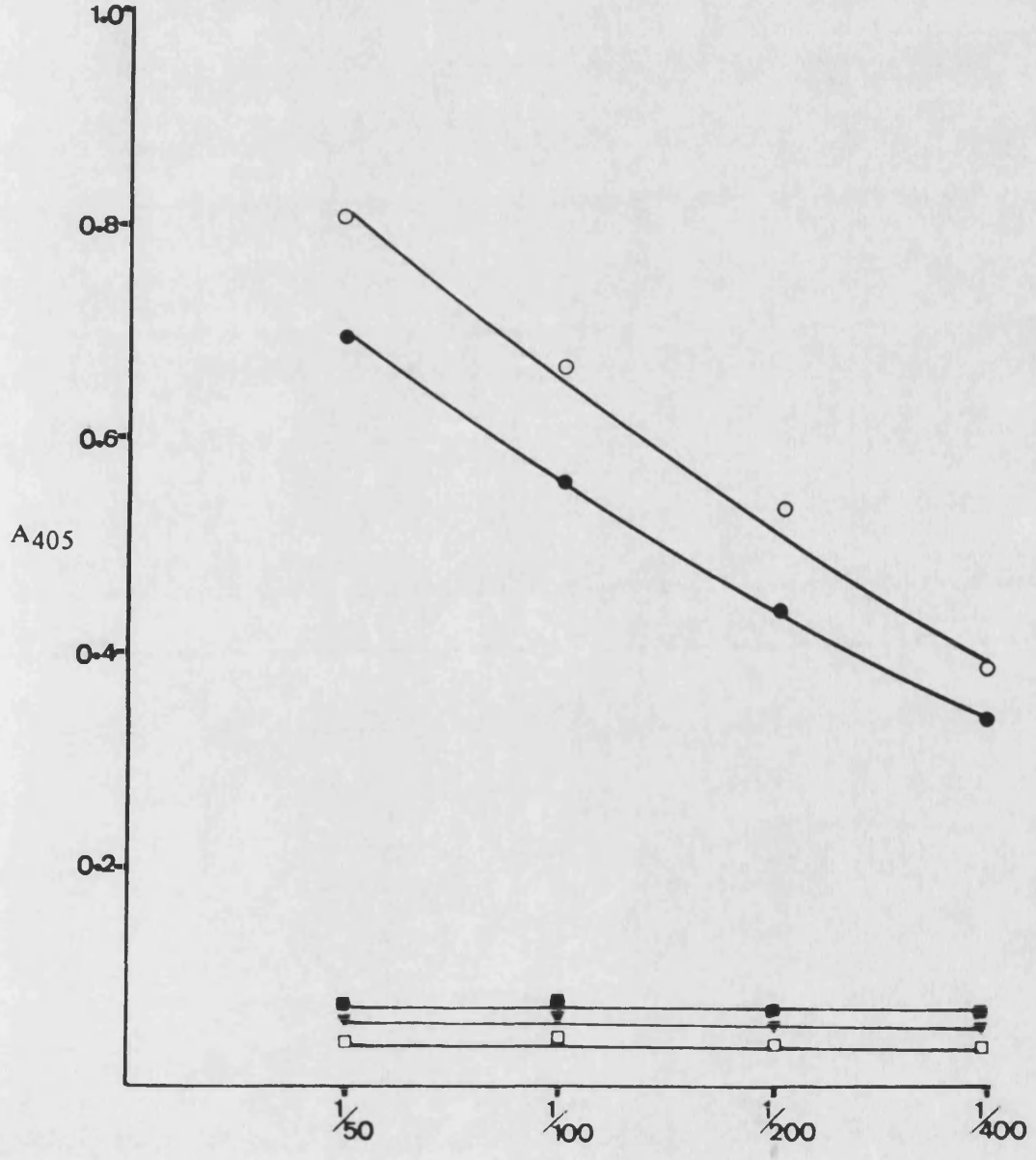


Fig. 6.4 Specificity of anti-peptide sera in direct ELISA against PCS-2

Substrate incubation was for 45min.

(■ , □) anti PCS-2 in both rabbits, (●) anti-PCS-1, (○) anti-KLH, (▼) normal rabbit serum. Each point represents the mean of duplicate determinations.

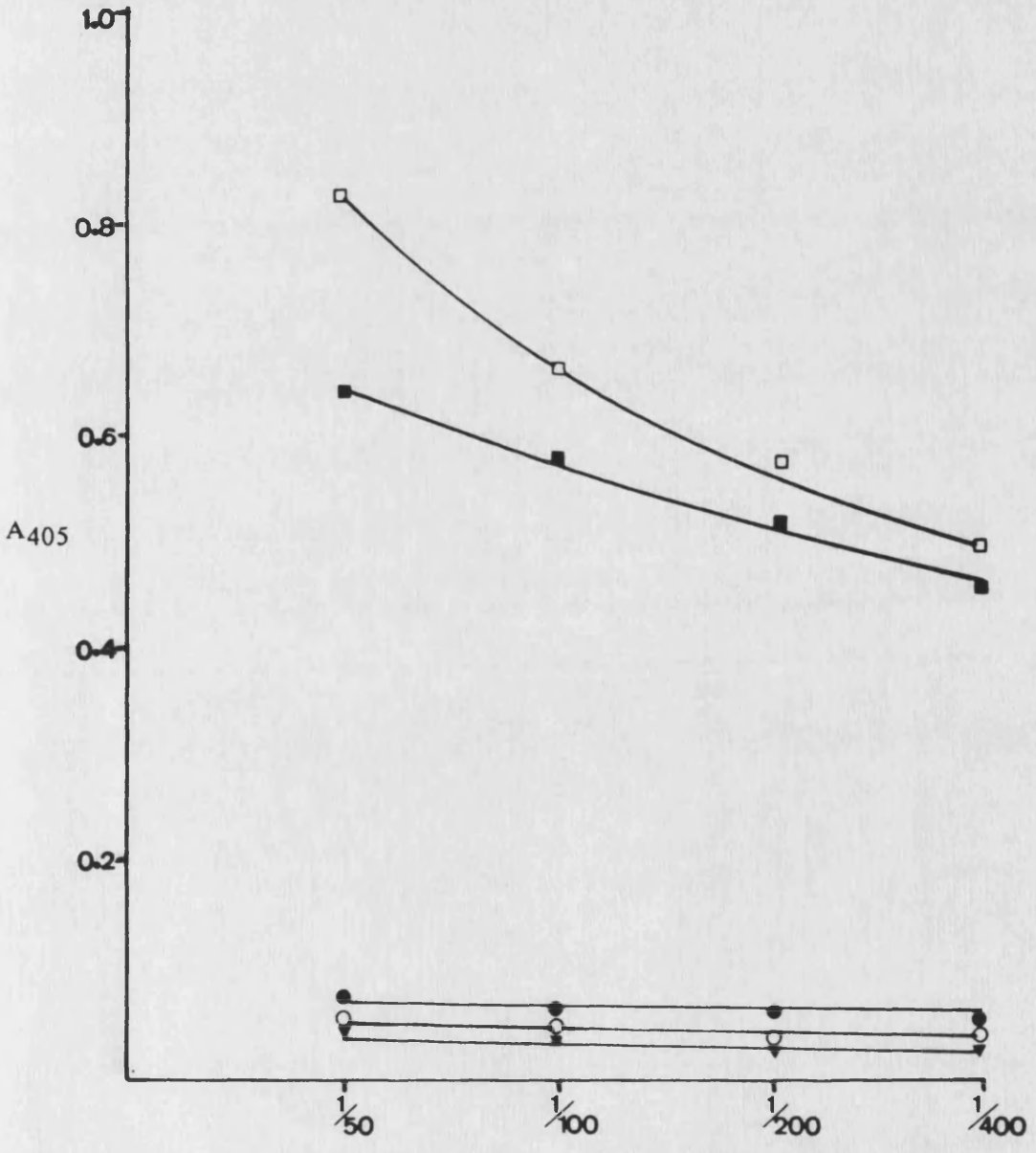
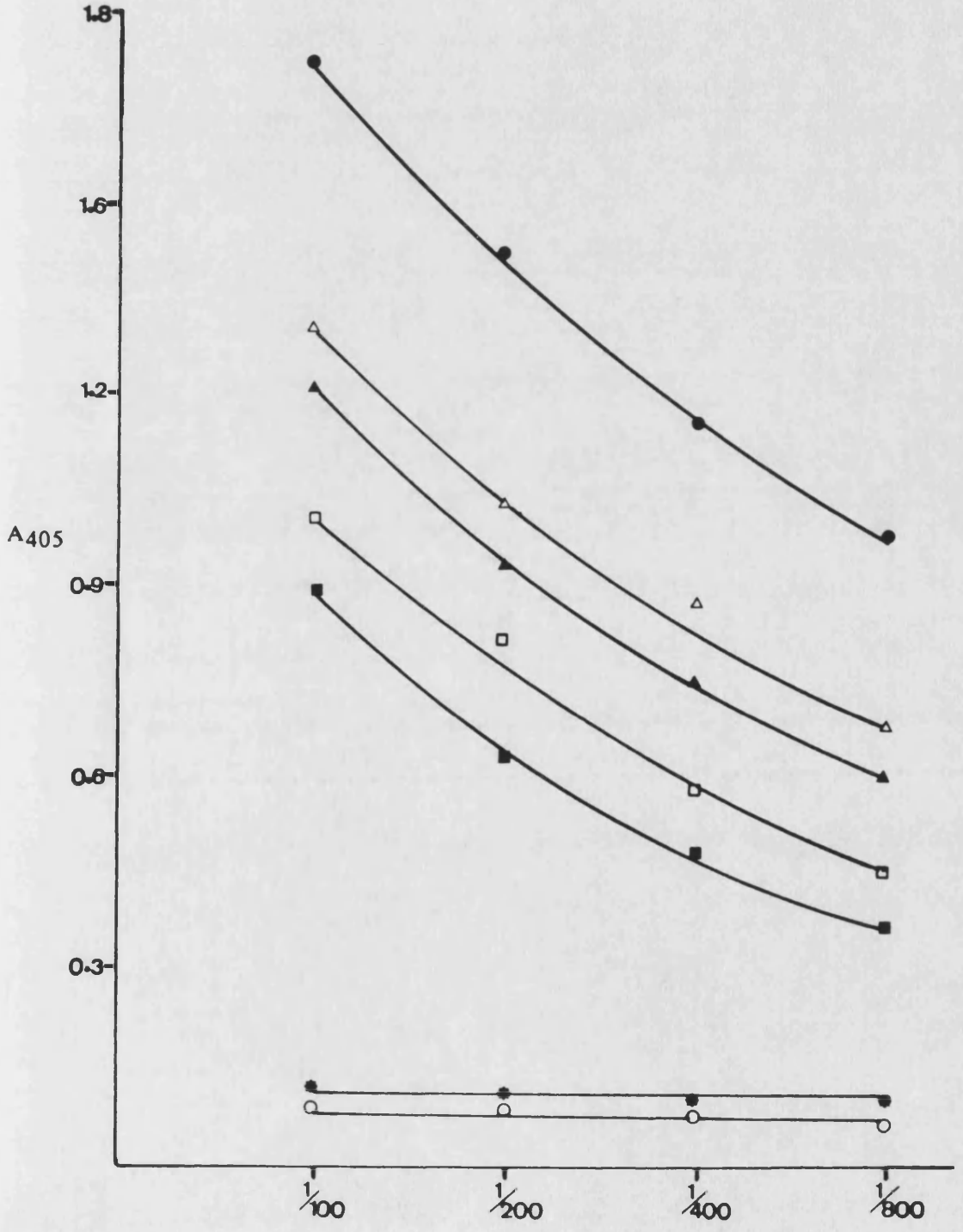


Fig. 6.5 Dilution curves for anti-PHCS and anti-peptide sera in direct ELISA against PHCS

Substrate incubation was for 1h.

(●) anti-PHCS, (△,▲) anti-PCS-1, (□,■) anti-PCS-2, (✱) anti-KLH,

(○) normal rabbit serum. Each point represents the mean of duplicate determinations.



6.4 REACTIVITY OF ANTI-PEPTIDE SERA WITH EXTENDED PEPTIDE

Peptides PCS-1 and PCS-2 were both extended with two amino acid residues (gly and cys) for coupling to carrier for immunizing, as described in section 3.6. Therefore, the extended peptides (PCS1-GC and PCS2-GC) were used to examine their interaction with anti-PCS-1 and anti-PCS-2 sera using the direct ELISA technique. The plates were coated with the antigens PCS-1, PCS1-GC, PCS-2 and PCS2-GC at 1μg/ml and then were exposed to either anti PCS-1 or anti PCS-2 at a series of dilutions. After that, assays were carried out as described in section 3-7a.

The results obtained are shown in Fig. 6-6 and Fig. 6-7. The results show that the anti-peptide sera react with the corresponding peptide in either extended or non-extended form.

That is, anti peptide PCS-1 sera reacted with both PCS-1 and PCS1-GC but not with PCS-2 and PCS2-GC and vice versa. Both anti PCS-1 and anti PCS-2 showed higher reactivity with their extended peptides than with the corresponding non-extended peptide.

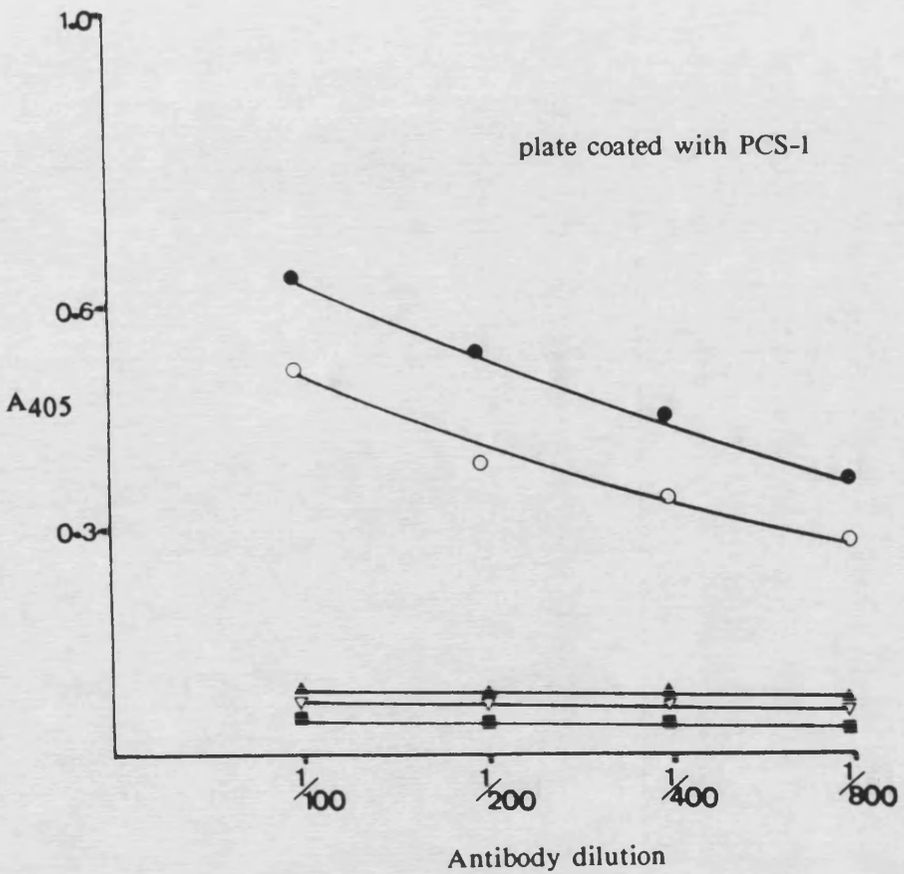
6.5 INHIBITION OF ANTI-PEPTIDE SERA BY PEPTIDES

Inhibition by peptide of anti-peptide serum binding to solid-phase antigen was examined using the competitive ELISA technique. The anti-peptide serum at a fixed dilution (1:100) was incubated with varying concentrations of peptide (0, 0.1, 0.5, 1, 5 and 10 μg/ml) for 1 h at 37°C prior to transfer to the wells of plate coated with PHCS. Then the assay was continued as described in section 3.7.

Plotting the percentage of anti-peptide bound versus the competing antigen added gave the results shown in Fig. 6.8 and 6.9. From Fig. 6.8 it can be seen that

Fig. 6.6 Reactivity of anti-PCS-1 against PCS-1 and PCS 1-GC in direct ELISA

Substrate incubation was for 40min. (●,○) anti-PCS-1, (▲), anti-PCS-2, (▽) anti-KLH, (■) normal rabbit serum. Each point represents the mean of duplicate determinations.



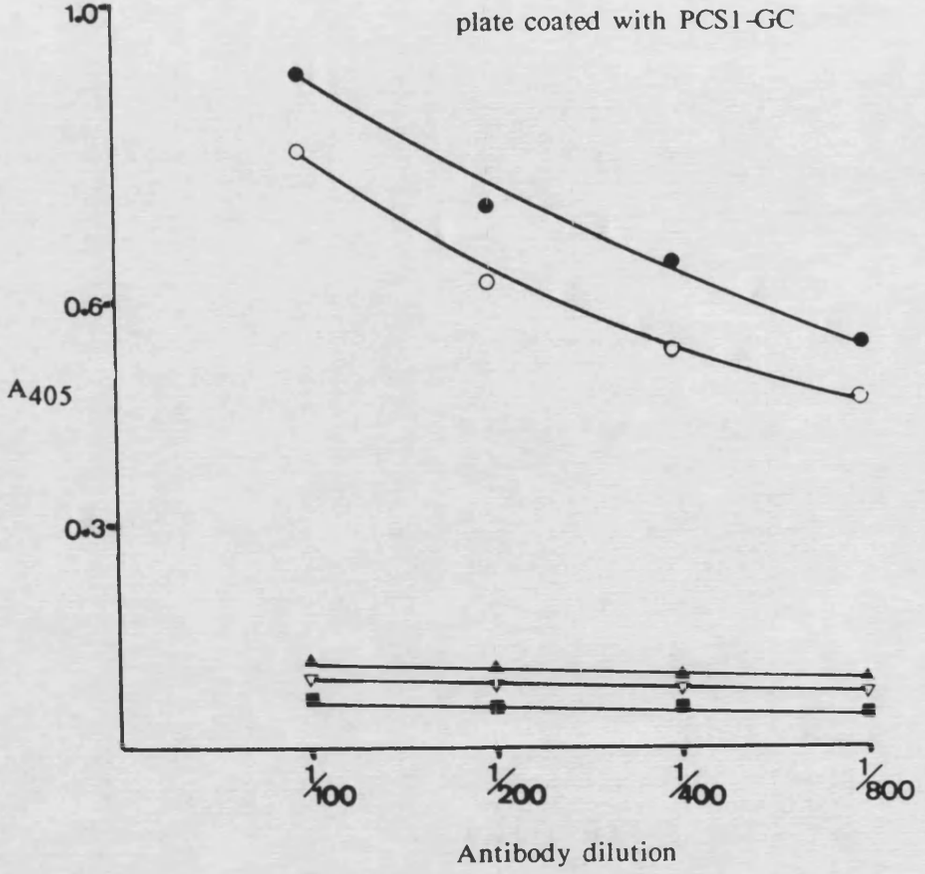
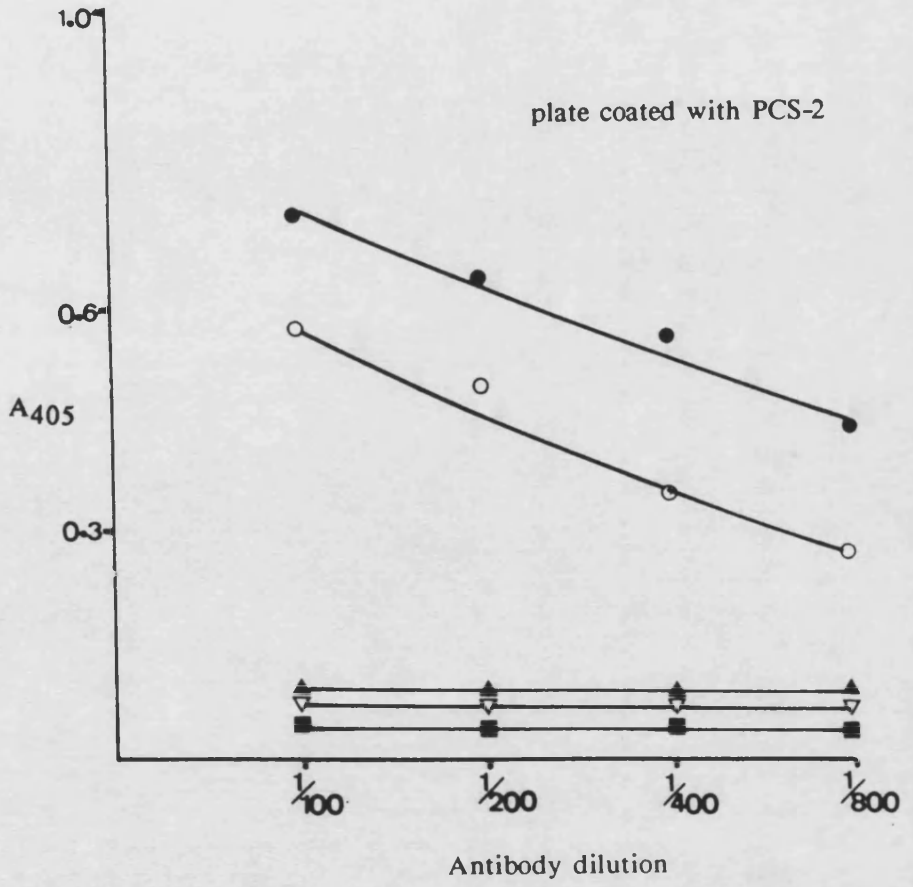
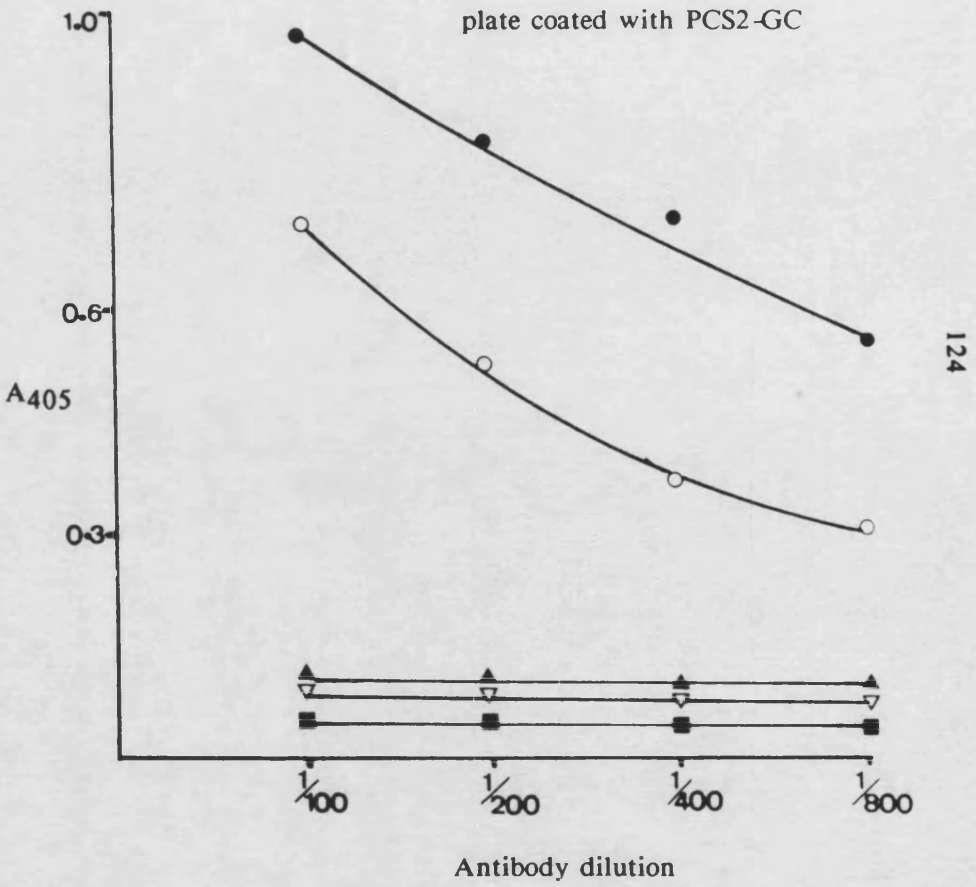


Fig. 6.7 Reactivity of anti-PCS-2 against PCS-2 and PCS2-GC in direct ELISA

Substrate incubation was for 40min. (●,○) anti-PCS-2, (▲) anti-PCS-1, (▽) anti-KLH, (■) normal rabbit serum. Each point represents the mean of the duplicate determinations.





anti-PCS-1 was inhibited partially using peptide PCS-1 as competing antigen. The maximum inhibition is about 22%, while the same anti-peptide was fully inhibited by the extended peptide PCS1-GC which gave 83% inhibition.

In the case of anti-PCS-2, from Fig. 6-9 it can be seen that both peptide PCS-2 and the extended peptide PCS2-GC fully inhibited the anti-PCS-2, giving 83% and 89% inhibitions, respectively.

6.6 INHIBITION OF ANTI-PEPTIDE SERA BY PHCS

The same technique was used in this assay as in the previous section 6.5, except that the anti-peptide sera (PCS-1 and PCS-2) were incubated with varying concentrations of native enzyme (PHCS) (0, 2, 5, 10, 20 and 50 μg/ml) for 1 h at 37°C, prior to transfer to wells of plate coated with 150 μl of PHCS (10 μg/ml).

Plots of absorbance at 405 nm against the concentration of antigen (PHCS) added are shown in Fig. 6.10 for both anti-PCS-1 and anti-PCS-2; normal rabbit serum was used as control. It can be seen from Fig. 6.10, in the case of using anti-PCS-1, that there is a region termed "prozone" which was found in contrast to the result obtained with anti-PCS-2. This phenomenon might be explained by the formation of soluble immune-complexes. Binding of the immune-complexes to the plate could result in an increase in the amount of the enzyme-conjugate bound.

Fig. 6.11 shows the percentage of anti-peptide bound as a function of the competing antigen (PHCS) added. It can be seen that anti-PCS-1 was slightly blocked by PHCS and gave a maximum percentage inhibition of about 26%, but anti PCS-2 was blocked to a greater extent by PHCS, and the maximum percentage inhibition was around 53%.

Fig. 6.8 Inhibition of anti-PCS-1 by PCS-1 and PCS1-GC in competitive direct ELISA

Substrate incubation was for 1h, (●) with PCS-1, (▼) with PCS1-GC, (□) normal rabbit serum. Each point represents the mean of duplicate determinations.

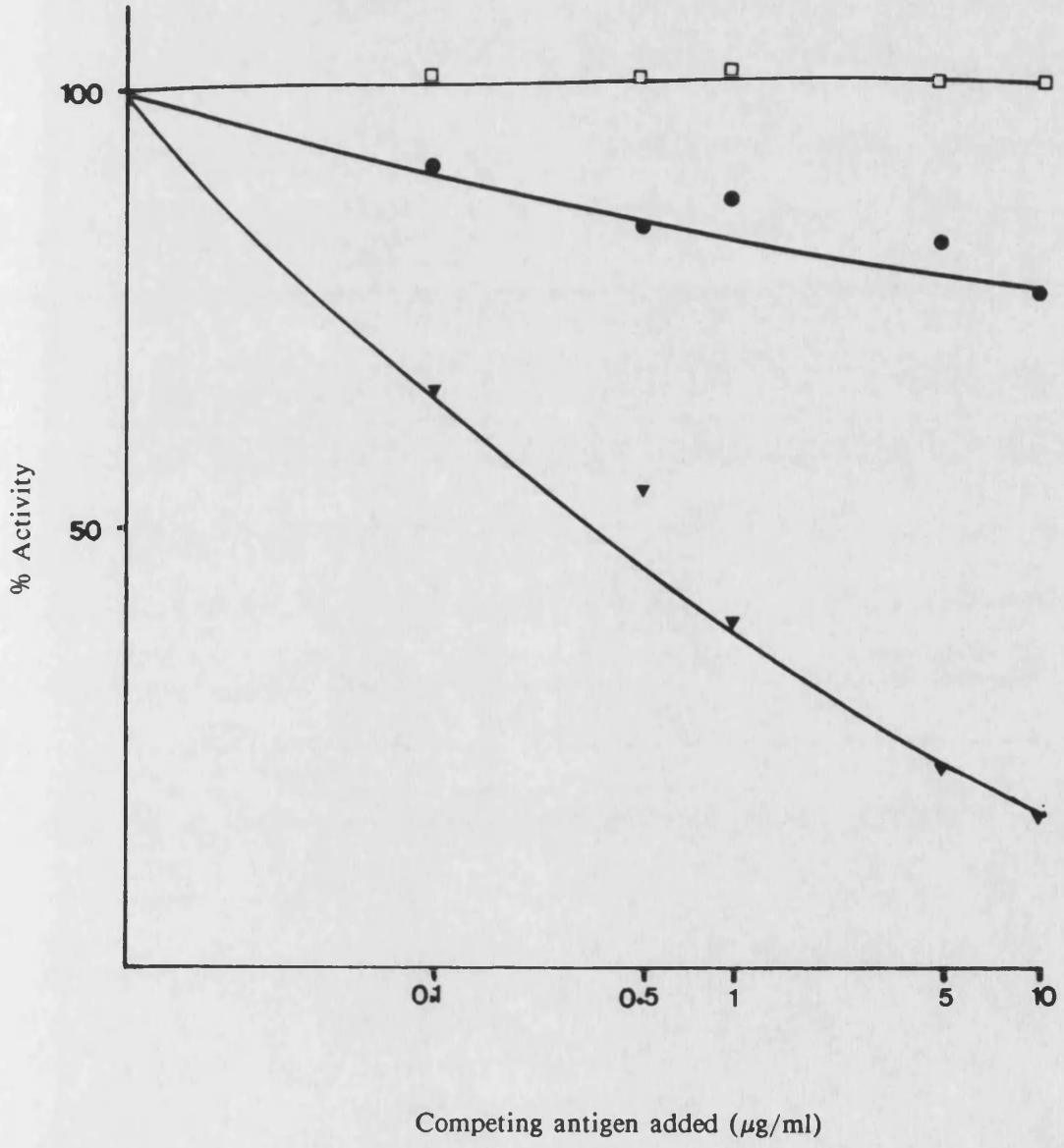


Fig. 6.9 Inhibition of anti-PCS-2 by PCS-2 and PCS2-GC in competitive direct ELISA

Substrate incubation was for 1h, (▽) with PCS2-GC, (○) with PCS-2, (□) normal rabbit serum. Each point represents the mean of duplicate determinations.

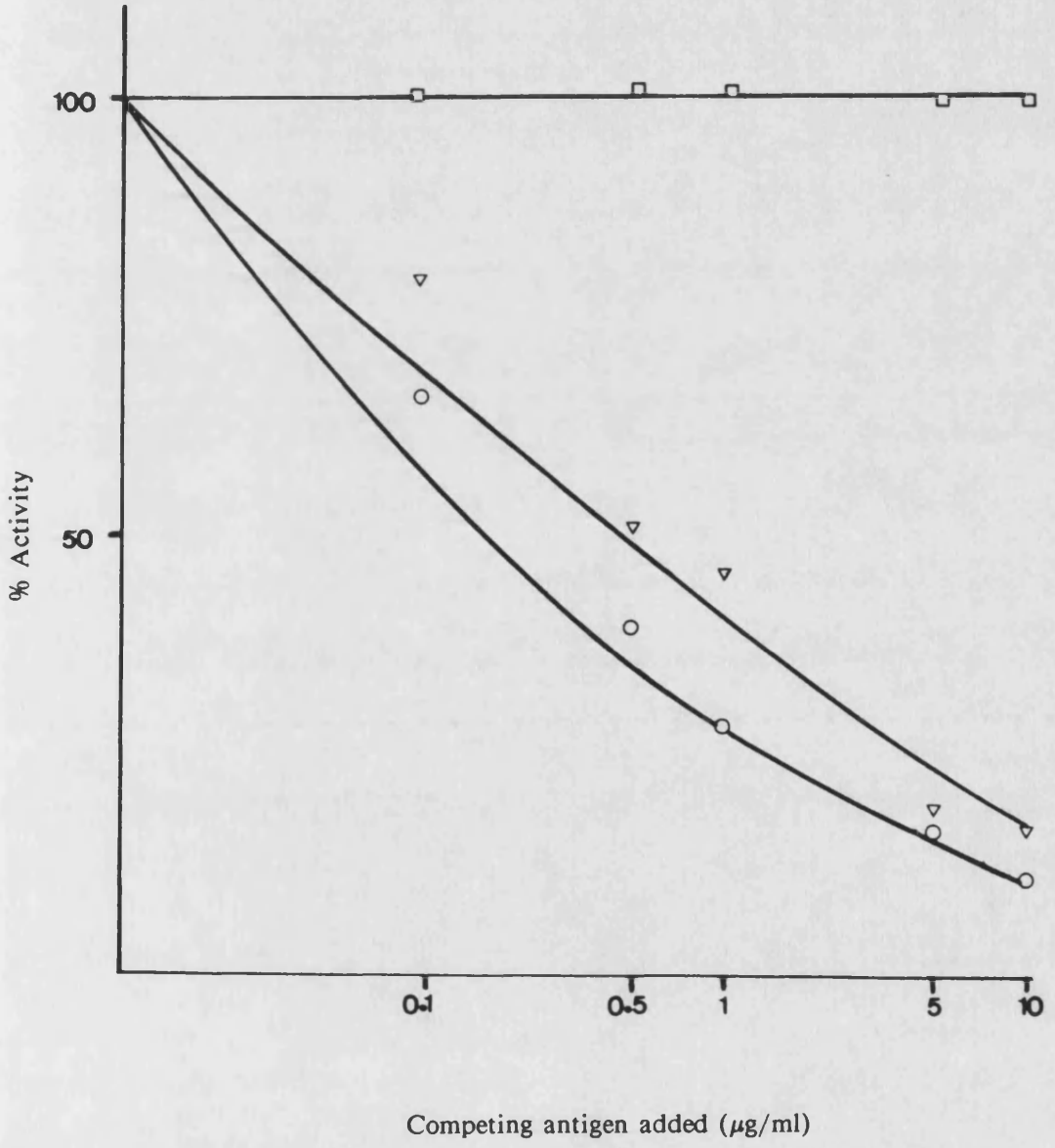


Fig. 6.10 Inhibition of anti-peptide sera by soluble PHCS in competitive direct ELISA

Substrate incubation was for 45min, (●) anti- PCS-1, (▲) anti-PCS-2, (□) normal rabbit serum.

Each point represents the mean of duplicate determinations.

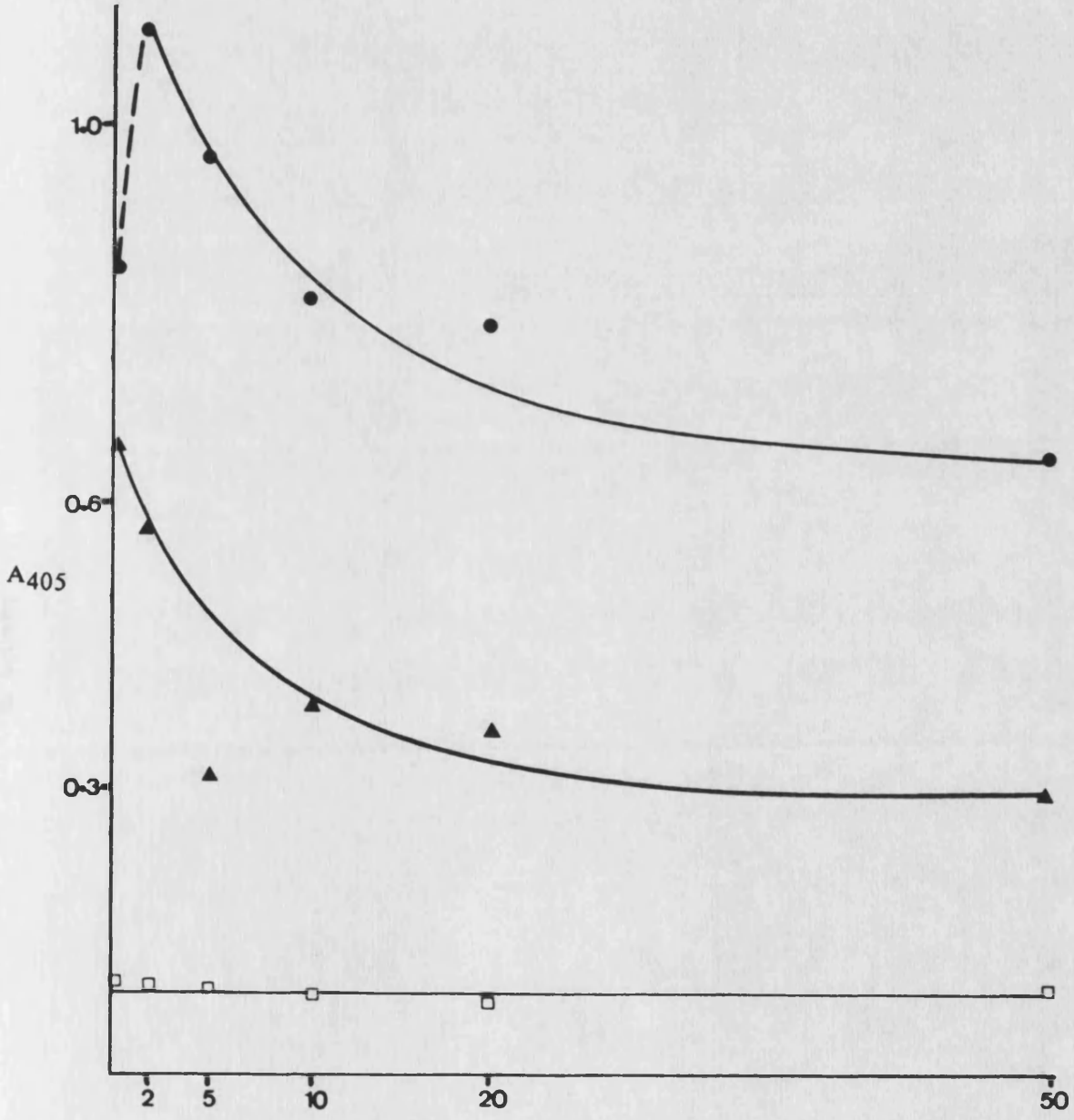
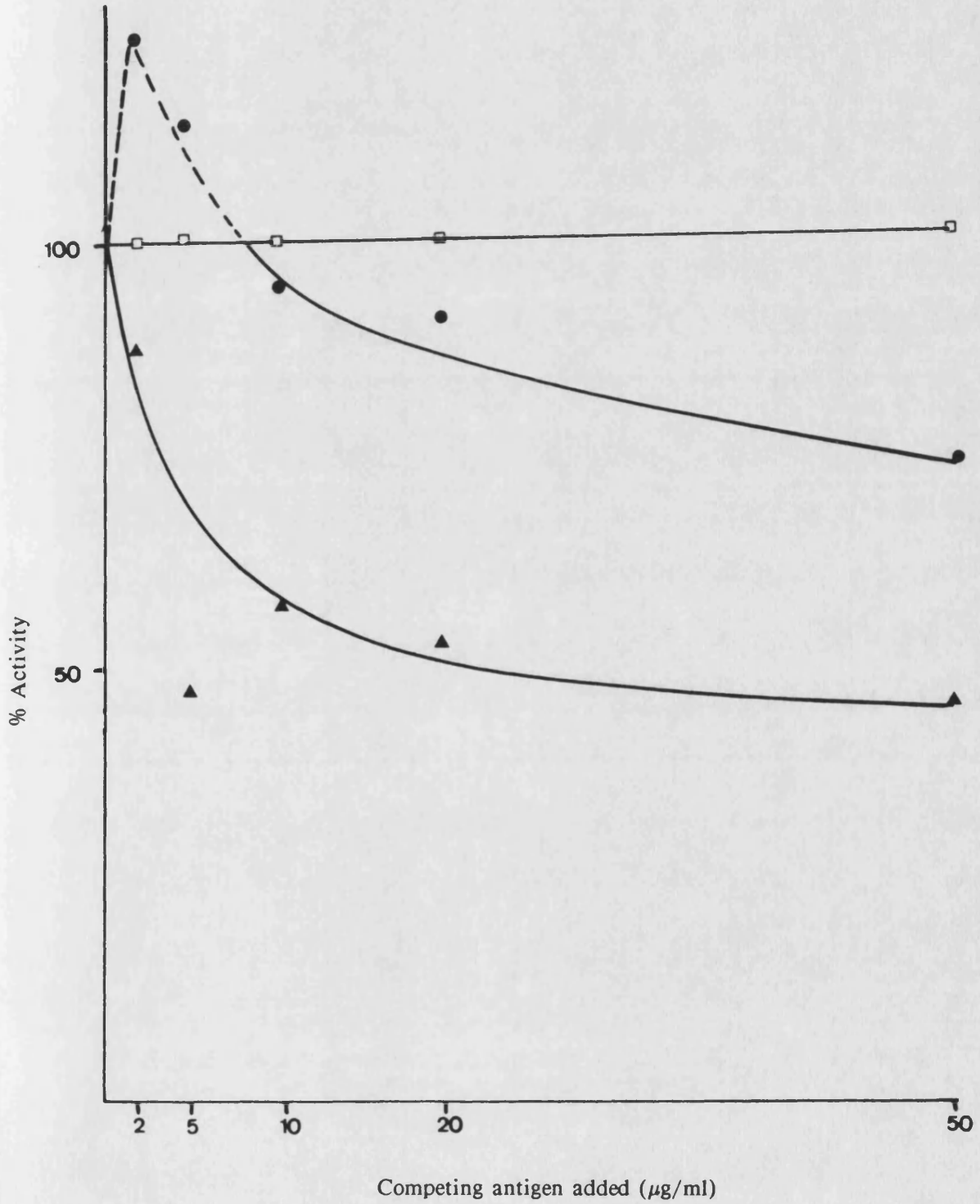


Fig. 6.11 Inhibition of anti-peptide sera by soluble PHCS

for details see Fig. 6.10



6.7 REACTIVITY OF ANTI-PEPTIDE SERA WITH CS FROM DIFFERENT SPECIES

Antisera against peptides PCS-1, PCS-2 and against native PHCS were tested for their cross-reactivity with citrate synthase from different species. Citrate synthase from pig heart, E. coli and A. anitratum, the only purified CSs available, were examined using direct ELISA.

The plate was coated with antigen (10 μg/ml) of different species, then each antigen was incubated with a range of dilutions of each antibody. The assays were carried out as described in section 3.7. The results obtained, plotted as absorbance at 405nm against antibody dilution, are shown in Fig. 6.12. It can be seen that only the citrate synthase of pig heart reacted with the anti sera, anti-PCS-1, anti-PCS-2 and anti-PHCS. The citrate synthases of the other two species showed no significant reactivity with both anti-peptide sera, as might be expected due to the difference in amino acid sequence between these enzymes in the regions corresponding to peptides PCS-1 and PCS-2 as shown in Fig. 6.13. Anti-PHCS sera showed very slight reactivity with these two enzymes, possibly due to homology in amino acid sequences at other regions of these enzymes.

6.8 DOT-BLOT IMMUNOSTAIN

The anti peptide sera, anti PCS-1 and anti PCS-2 were tested for their reactivity with native PHCS using the dot-blot immunostain technique. The nitrocellulose sheets were cut and spotted with aliquots (2μl) of PHCS of varying concentrations (0.1, 0.2, 1, 10 μg/ml in PBS) and then incubated with anti PCS-1 sera, anti PCS-2 sera (diluted 1/50 and 1/100) and anti PHCS sera (diluted 1/100). The experiment was then carried out as described in section 3.17. The nitrocellulose sheets incubated with

Fig. 6.12 Cross-reactivity of anti-PHCS and anti-peptide sera with CS from different species in direct ELISA
Substrate incubation was for 45min.
Anti-PHCS (■), anti-PCS-1 (○) and anti-PCS-2 (▲) were reacted with CS from pig heart (1), E. coli (2) and A. anitratum (3). (□) represents the normal rabbit serum control. Each point represents the mean of duplicate determinations.

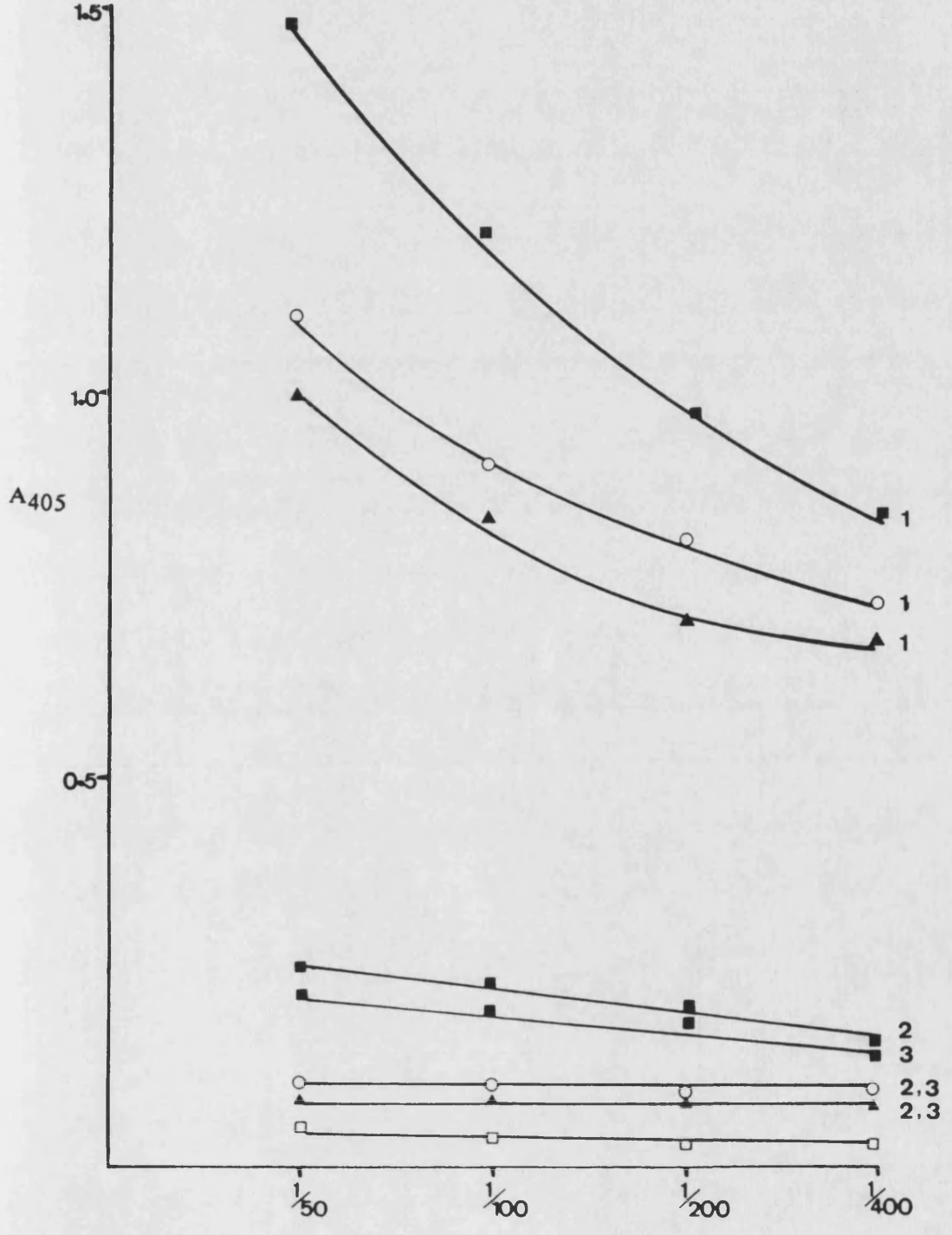


Fig. 6.13: The amino acid sequence of peptides PCS-1 and PCS-2 of PHCS and the corresponding region of the CS sequence from E. Coli and A. anitratum

PCS-1 (288-302)

	288														302
PHCS	L	Q	K	E	V	G	K	D	V	S	D	E	K	L	R
E. Coli CS	I	S	S	-	V	K	H	-	I	P	-	E	F	F	R
A.anitratum CS	I	G	S	-	V	E	N	-	V	A	-	E	F	M	E
					★							★			

PCS-2 (76-90)

	76														90
PHCS	K	M	L	P	K	A	K	G	G	E	E	P	L	P	E
E. Coli CS	T	D	S	N	-	Y	-	-	-	-	-	-	L	-	E
A.anitratum CS	T	Q	A	D	-	Y	-	-	-	-	-	-	L	-	E
													★		★

anti-peptide sera showed varying degrees of reactivity of the antisera corresponding to their dilution and the concentration of antigen, as shown in Fig. 6.14, 6.15 and 6.16. The nitrocellulose sheet incubated with anti PHCS showed higher reactivity than those incubated with anti-peptide sera, particularly at the highest concentration of antigen used (10 μg/ml), as shown in Fig. 6.16. The results are summarised in Table 6.1.

6.9 IMMUNOBLOTTING OF NITROCELLULOSE-BOUND FRAGMENT OF PHCS

The fragmentation of PHCS by using CNBr was achieved as explained earlier in section 5.9b and four major fragments were produced (Fig. 5.15).

After that, electrophoretic transfer of proteins from the gel to nitrocellulose was achieved as described in section 3.14. Immunodetection of nitrocellulose-bound fragments of PHCS was performed as in section 3-16, using anti-peptide sera and rabbit anti-PHCS serum diluted 1/50 in 1% casein/PBS.

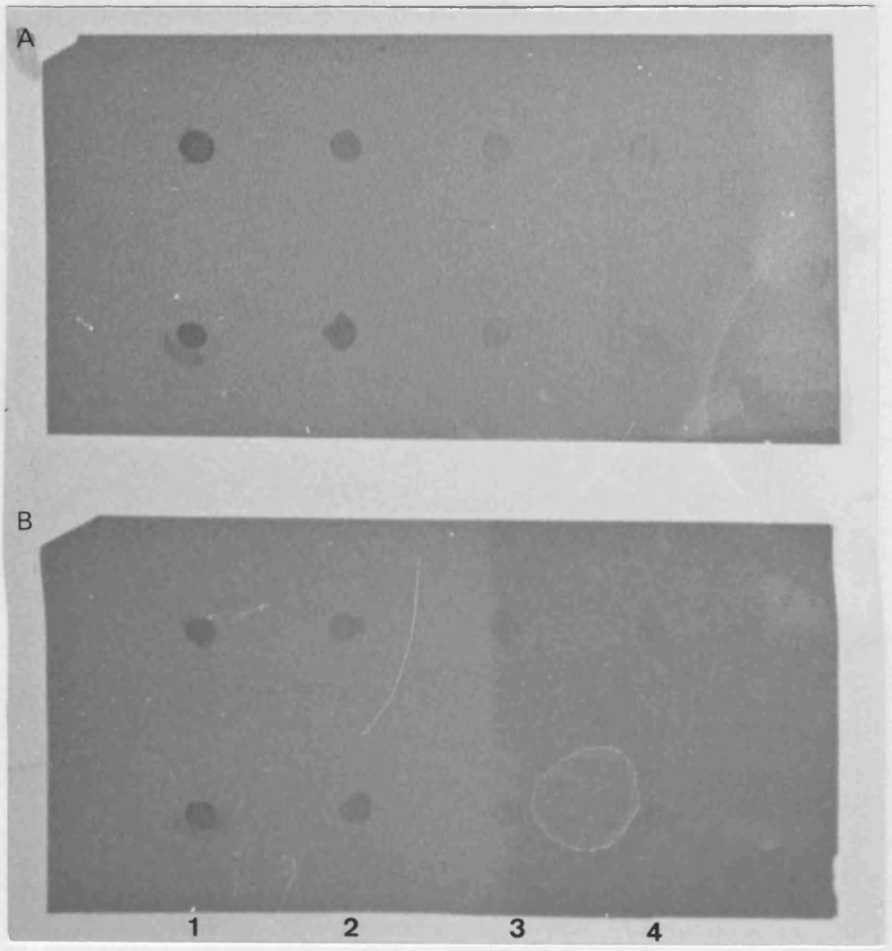
In this experiment, 4 gel tracks, (high molecular weight markers, low molecular weight markers, undigested PHCS and CNBr-fragmented PHCS, respectively) were stained with 0.025% (w/v) coomassie blue-R (in 7% (v/v) acetic acid) as described in section 3.13.

Following transfer to nitrocellulose six gel tracks were immunostained. Two tracks (undigested PHCS and fragmented PHCS) were incubated in separate containers with anti PCS-1, anti PCS-2 and anti PHCS sera.

From the graph shown earlier (Fig. 5.16), data obtained from the stained gel gave mol.wt. of fragments produced as 15.8K, 10K, 5.5K and 1.8K.

The reactivities of anti-sera following immunoblotting of PHCS fragments are shown in Fig. 6.17. A summary of these reactivities is shown in Table 6.2. The intensity of staining of each band in the gel, was visually rated on a scale of 1-5.

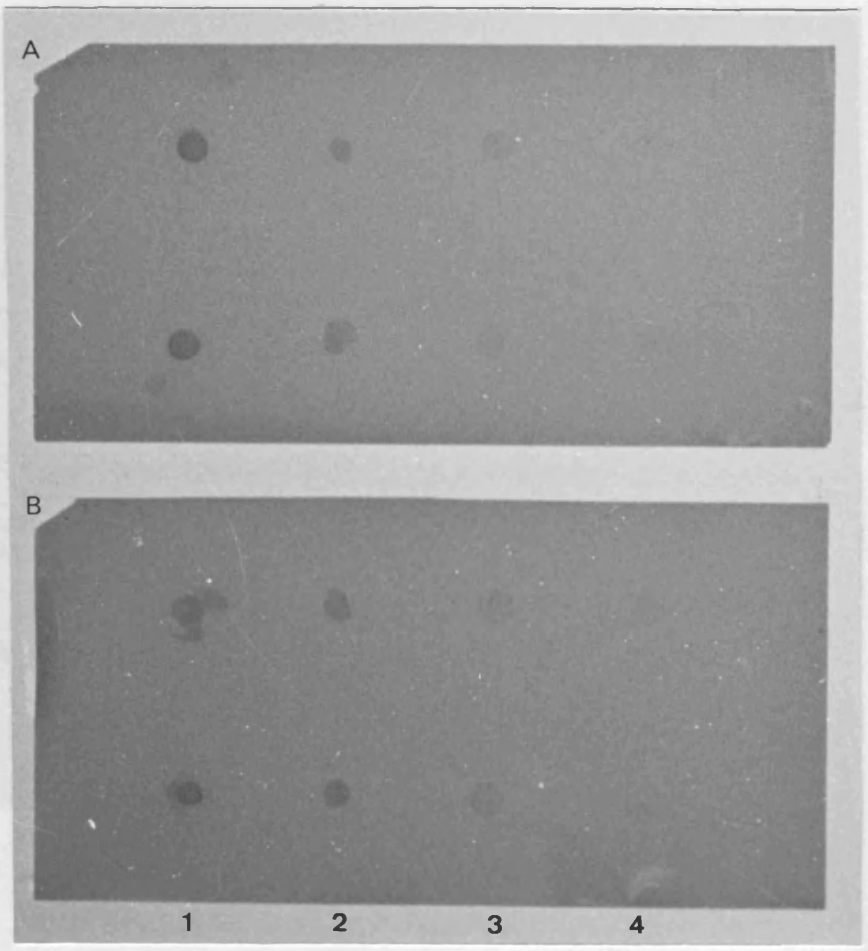
Fig. 6.14 Dot-blot immunostain of PHCS with anti-PCS-1 serum diluted 1:50 (A) and 1:100 (B)



Each concentration in duplicate

Spot No.	concentration of PHCS (μg/ml)
1	10
2	1
3	0.2
4	0.1

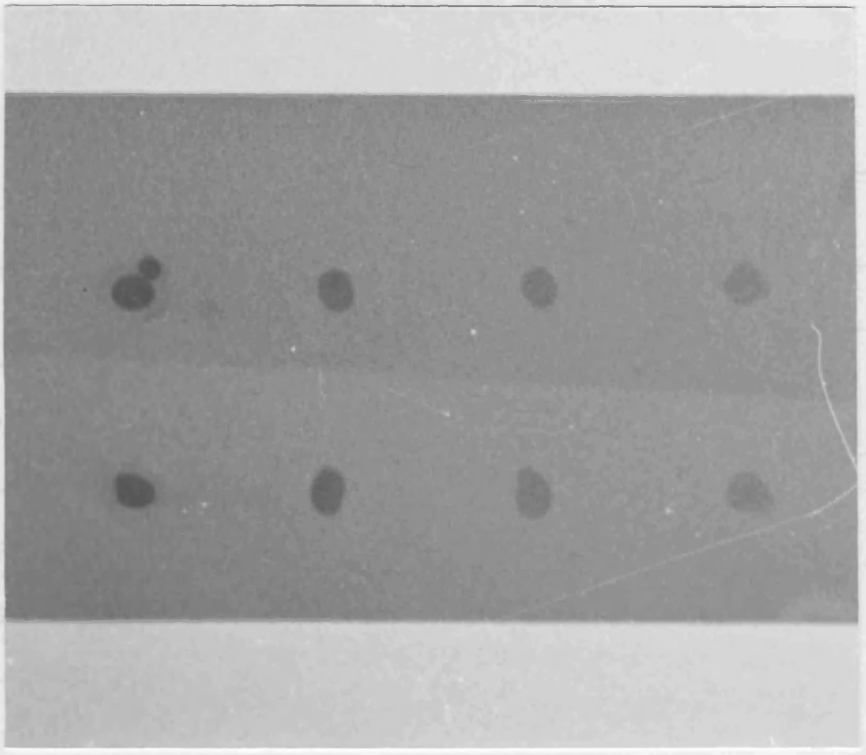
Fig. 6.15 Dot-blot immunostain of PHCS with anti-PCS-2 serum diluted 1:50 (A) and 1:100 (B)



Each concentration in duplicate

Spot No.	concentration of PHCS (μg/ml)
1	10
2	1
3	0.2
4	0.1

Fig. 6.16 Dot-blot immunostain of PHCS with anti-PHCS serum diluted 1:100



1 2 3 4

Each concentration in duplicate

Spot No.	concentration of PHCS (μg/ml)
1	10
2	1
3	0.2
4	0.1

Table 6.1 Dot-blot immunostain of anti-PHCS, anti-PHC-1 and anti-PCS-2 with native PHCS

Spot intensity was assessed visually on scale 0-5

anti sera	PHCS conc. (μg/ml)			
	10	1	0.2	0.1
anti PHCS (1:100)	5	4	3	3
anti PCS-1 (1:50)	3	2	1	1/2
anti PCS-1 (1:100)	2	1	1	0
anti PCS-2 (1:50)	3	2	1	0
anti PCS-2 (1:100)	2	1	1	0

The most intense band was assigned a value of 5 and the least intense a value of 1.

The results show that anti-PHCS detected a larger number of bands than were detected by anti-PCS-1 or anti-PCS-2. Anti-PCS-1 detected a band of mol.wt. 10.5K and additional higher mol.wt. bands. This band was not detected by anti-PCS-2, which detected a band of mol.wt. 8.3K and additional higher mol.wt. bands.

6.10 EFFECT OF ANTISERA ON PHCS ACTIVITY

a) Effect of antisera alone

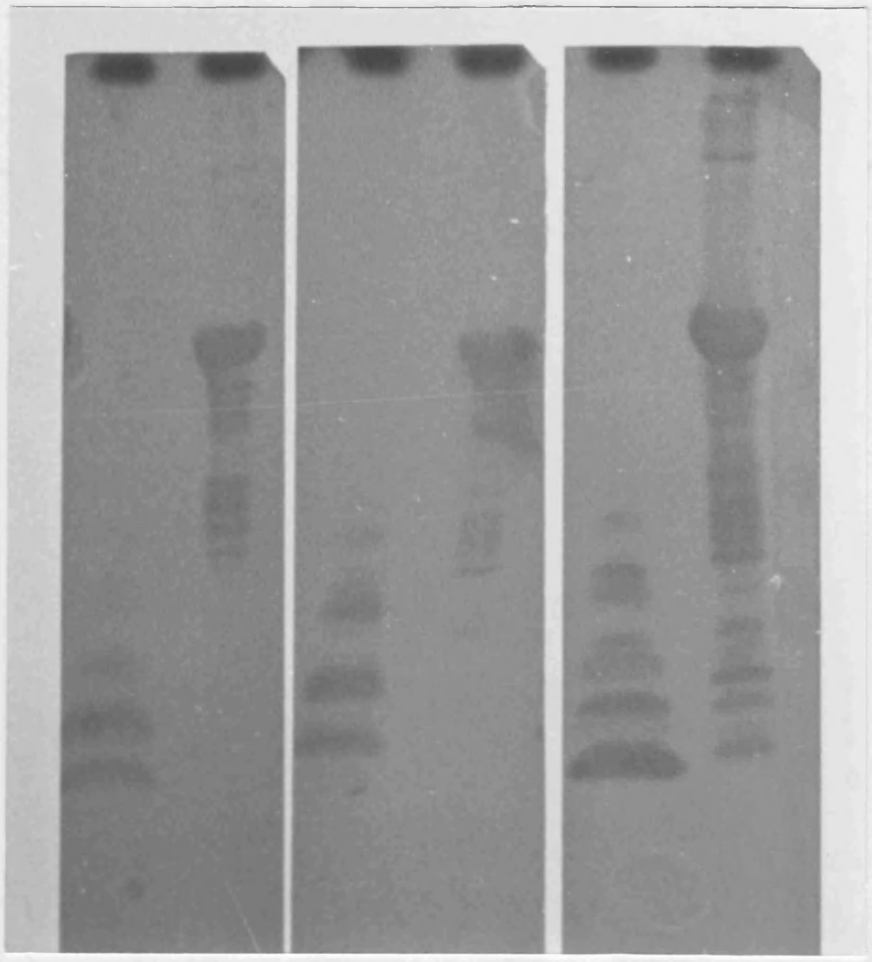
The pure enzyme (PHCS) was diluted in 20 mM tris-HCl/1mM EDTA buffer, pH 8.0, so that 10 μl aliquots would give a suitable reaction rate (0.04 units/min) in the assay described in section 3.18.

To investigate the effect of antisera on enzyme activity, 10 μl samples of suitably diluted enzyme solution were incubated with anti sera (anti-peptide sera, anti PHCS sera and normal rabbit sera) at varying dilutions or with varying concentration of IgG's of those sera, as described in section 3.18a.

Those antibodies that recognise and bind to epitopes within or near the active site should affect enzyme activity. This may be due either to the antibodies blocking the entry of substrates to the active site, or alternatively causing a conformational change "either enhancing or decreasing activity" as a result of their binding.

The results of the effect of anti-sera on the PHCS activity are shown in Fig. 6.18 by plotting the % activity versus amounts of antisera added. From Fig. 6.18 it can be seen that anti-PHCS sera showed a significant effect on the enzyme activity, while anti PCS-1 and anti PCS-2 sera showed no significant effect.

Fig. 6-17 PHCS fragmented at met residue by CNBr after immunoblotting with anti-peptide serum and anti-PHCS serum



1 2 3 4 5 6

Tracks 2,4,6; undigested PHCS. Immunoblots performed with anti-PCS-2 (track 2), anti-PCS-1 (track 4), and anti-PHCS serum (track 6).

Tracks 1,3,5; CNBr-digested PHCS. Immunoblot performed with anti-PCS-2 (track 1), anti-PCS-1 (track 3), and anti-PHCS serum (track 5).

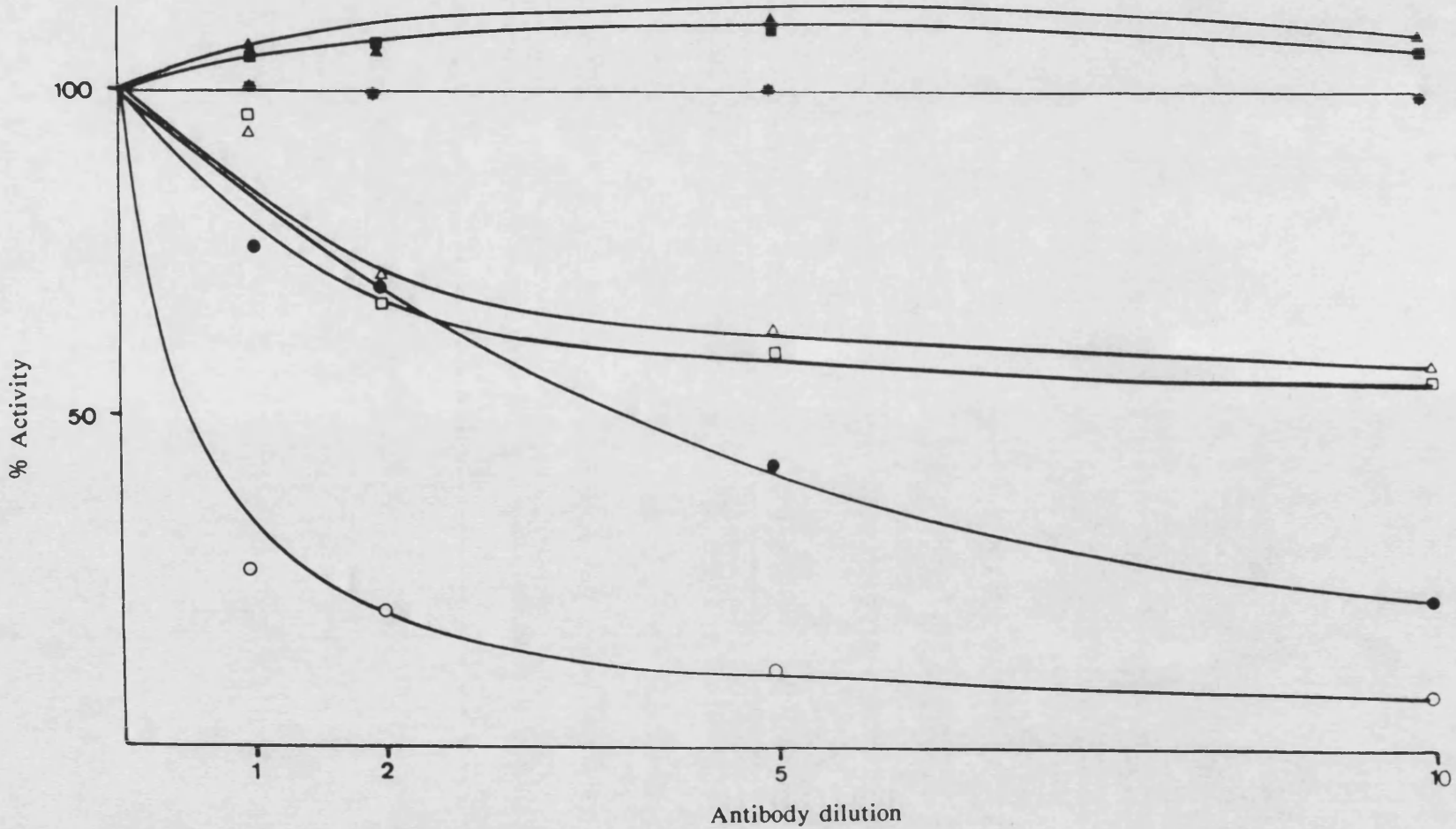
Table 6.2 Summary of the reactivity of anti-peptide sera and anti-PHCS serum with CNBr-digested PHCS

Fragment	Mol. Wt. (K)	Intensity of stain band with anti-PHCS	anti-PCS-1	anti-PCS-2
a	33.8	-	1	-
b	33.1	1	2	-
c	28.8	3	1	-
d	25.1	2	2	1
e	20.4	3	-	-
f	17.8	2	3	2
g	13.5	4	-	3
h	10.5	-	4	-
i	8.3	5	-	3
*j	5.5	-	-	-
*k	1.8	-	-	-

The intensity of the bands were assigned as mentioned in the text (section 6.9).

* These bands are seen on protein stain and are not detected after immunostaining.

Fig. 6.18 Effect of anti-PHCS and anti-peptide sera on enzyme activity before and after addition of protein A-Sepharose
Addition of (●) anti-PHCS, (▲) anti-PCS-1, (■) anti-PCS-2 and the open symbols after adding protein A-Sepharose (✱) normal rabbit serum.



b) Effect of antisera and Protein A

As pointed out in section 3.18b, incubation of the enzyme-antibody complexes with protein A, followed by centrifugation, can be used to detect all antibody binding to the enzyme, regardless of their fine specificity. So, the assays of enzyme activity in the presence of a suspension of insoluble protein A were carried out as described in section 3.18b. The results are shown in Fig. 6.18, by plotting the % activity against amounts of antisera added (including insoluble protein A). It can be seen from Fig. 6.18 that the use of anti-PHCS sera with protein A showed a greater effect on the enzyme activity than that without protein A. Also, both anti-peptide sera with protein A had an effect on the enzyme activity, whereas they did not show any significant effect without protein A.

6.11 DISCUSSION

From the results presented above, it can be concluded that specific antibodies against the synthetic peptides PCS-1 and PCS-2 were produced, when rabbits were immunized with peptides coupled to carrier-protein KLH. The antibody levels increased in the serum as the number of boosting injections was increased. Each antiserum reacted with the immunizing peptide, but not with the other peptide. Also, each antiserum reacted with either the extended or non-extended immunizing peptide but not with the extended form of the other peptide. However, anti-KLH sera or normal serum reacted with neither peptide, extended or non extended.

The reaction of anti-PCS-1 sera was partially inhibited by peptide PCS-1 (maximum inhibition was approximately 22%), and more strongly extended peptide PCS1-GC (83% inhibition), when PHCS was the immobilized target antigen in competitive ELISA assays. Anti PCS-2 serum was strongly inhibited by peptide PCS-2

and extended peptide PCS2-GC (83% and 89% inhibition respectively).

Anti-PCS1 sera is only slightly blocked by intact PHCS (26%) while anti-PCS-2 sera was inhibited by approximately 53%. We conclude that in each case part of the anti-peptide antibody population is directed against native determinants (blocked by soluble PHCS) and part of the antibody population is directed against denatured determinants on the solid phase antigen and can not be blocked by soluble, native enzyme. Anti-PCS-2 contains a greater proportion of anti-native state antibodies than anti-PCS-1.

When using citrate synthase from different species to detect their cross-reactivity with anti-peptides sera, there was no significant reactivity with E. coli "CS" and A. anitratum "CS"; the only reactivity was with PHCS.

This was not an unexpected result in that the amino acid sequence of pig heart CS shows little or no homology with E. coli or A. anitratum CS in the regions corresponding to peptides PCS-1 and PCS-2.

Results of immunoblot of nitrocellulose-bound CNBr-fragments of PHCS, agreed well with those of ELISA as both anti-peptide sera and anti-PHCS sera bind in varying degrees to fragments of PHCS. The intact PHCS was digested by CNBr and yielded four major fragments of molecular weights 15.8K, 10K, 5.5K and 1.8K, respectively, as discussed in section 5.9b.

From Table 5.3 (page 103), which shows the expected fragments of PHCS produced by treatment with CNBr, it can be seen that there is a major fragment between residues 267-346 of mol.wt. of 9.6K. The region corresponding to peptide PCS-1 (288-302) is contained in this fragment. So, the fragment of 10K mol. wt. yielded through digestion of the enzyme could be consistent with that fragment. The fragment between residues 78-127, of mol.wt. 6K, contained most the residues of PCS-2 (76-90), thus the fragment of 5.5 mol.wt. produced could be consistent with that fragment. The fragment of 15.8K mol.wt. may be as a result of partial degradation. Furthermore, the other observed fragment of 1.8K mol.wt., relevant to some fragments

in the Table 5.3.

The immunoblot of native PHCS using anti-PHCS shows evidence of degradation of the enzyme since several lower mol.wt. bands can be seen in addition to the expected 49K band. A similar result is seen when anti-PCS-1 or anti-PCS-2 are used.

Table 6.2 shows that anti-PCS-1 and anti-PCS-2 react with predicted fragments (approximately mol. wt. 10K and 6K respectively) together with additional higher mol. wt. bands which might result from incomplete cleavage pf PHCS by CNBr. This is confirmed by the reactivity of anti-PHCS with fragments of mol. wt. greater than 10K.

Anti sera to the synthetic peptides PCS-1 and PCS-2 did not show any significant effect on the assay of enzyme "PHCS" activity. Since both antisera were shown to bind intact PHCS by using the direct ELISA technique, as mentioned earlier, therefore binding of these antibodies to PHCS appears not to affect the activity of this enzyme. When the inhibition experiments were repeated with the addition of insoluble protein A in order to precepitate antibody-enzyme complexes, anti-PCS-1 and anti-PCS-2 were shown to have a significant effect on enzyme activity. This confirms that soluble complexes of PHCS with anti-PCS-1 or anti-PCS-2 retain their enzymic activity, and that this activity is lost as the complexes are precepitated with protein A.

CHAPTER SEVEN CONCLUDING REMARKS

7.1 STRATEGY

Since the experimental study of protein antigenicity is difficult and time consuming, it is of considerable importance to obtain theoretical procedures for the prediction of protein epitopes. This is of particular interest because many protein sequences are obtained from gene sequences of proteins which have not been purified or are not available in large amounts.

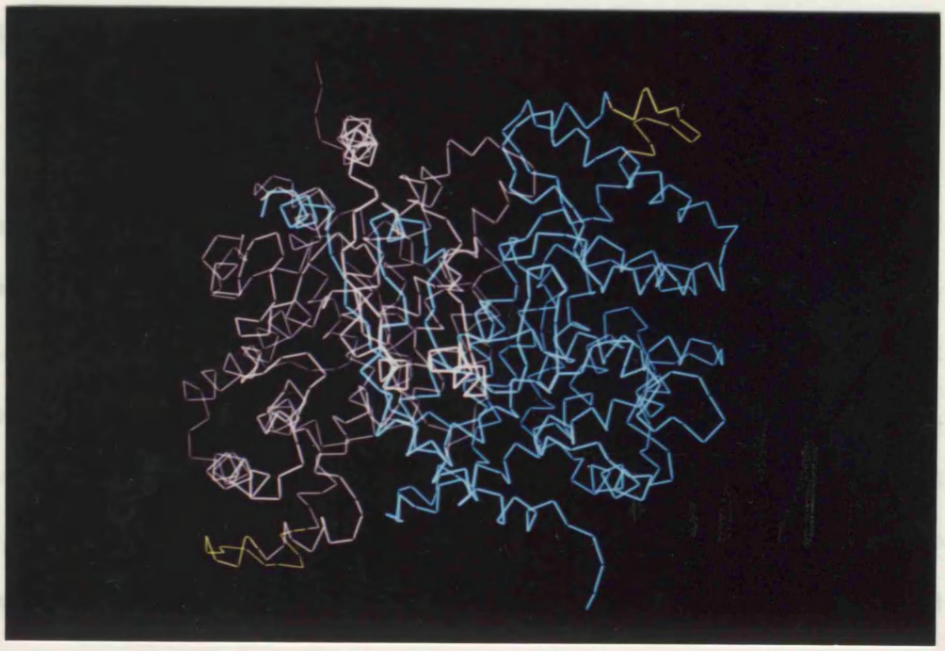
So in this study a number of predictive methods were used to determine potential antigenic and immunogenic peptide sequences of PHCS, including methods depending on the amino acid sequence alone (hydrophilicity and composite surface profile) and methods using conformation data from the crystallographic structure of the enzyme (protrusion and mobility). However, it is difficult to assign antigenicity with complete confidence using any one parameter, although there is strong correlation between some of them.

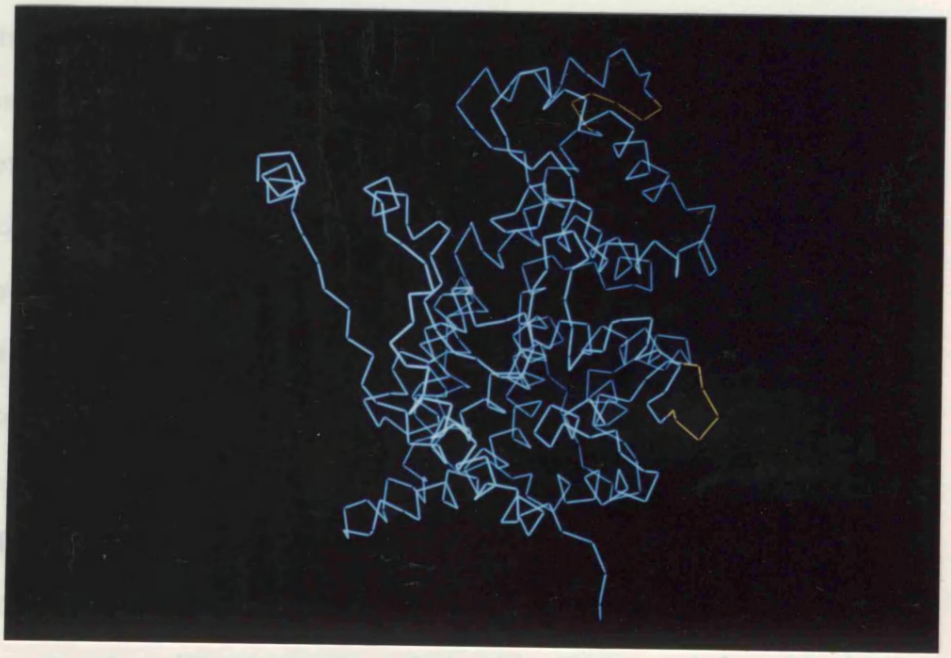
According to these methods, two peptides which are thought to be important in the antigenic activity of PHCS were selected and synthesized, peptide PCS-1 at region 288-302 and peptide PCS-2 at region 76-90. PCS-1 is contained in a region which seems to be on the external surface of the enzyme molecule, as can be seen from the structural model (Fig. 7.1a). It was found that both groups of predictive methods, those based on the amino acid sequence or the conformational structure, indicate that the region 288-302 could be strongly antigenic. The region containing the PCS-2 peptide, residues 76-90, is highly predicted from a consideration of mobility (structure based), although this segment appears to be non-protrusive. PCS-2 is also predicted less reliably using sequenced-based methods. The location of PCS-2 peptide also seems to be on the external surface of the (PHCS) molecules as seen from the structural model (Fig. 7.1b), at a distance from, and not overlapping with, the PCS-1 peptide.

Fig. 7.1 Location of synthetic peptides

1a) CS dimer showing monomers coloured blue and pink. The location of peptide PCS-1 (288-302) is indicated in yellow on each subunit.

1b) CS monomer showing the approximate location of peptides PCS-1 (288-302) and PCS-2 (76-90). The regions coloured yellow represent residues 70-85 and 290-295.





7.2 CROSS-REACTIVITY OF POLYCLONAL AND MONOCLONAL ANTIBODIES TO INTACT ENZYME

Results presented in this study demonstrate that polyclonal anti-PHCS antibodies bind to intact PHCS as expected, and also to the two synthetic peptides PCS-1 and PCS-2. As indicated above, these peptides correspond to predicted epitopes of PHCS (regions 288-302 and 76-90, respectively). A library of MAbs, originally raised against PHCS, showed varying degrees of interaction with PCS-1 and PCS-2. Unexpectedly, most MAbs showed similar reactivity against PCS-1 and PCS-2, although these peptides clearly correspond to determinants which are located on different regions of the surface of native PHCS. However, the reactivity found between MAbs and a control peptide (x-peptide) suggests the possibility of non-specific interactions, which may be caused by conformational features of the peptides which may not correspond to their structures in the native molecule. Similar effects have been observed by Jemmerson (1987) who studied the binding of MAb to fragments of rat cytochrome C.

Clearly the use of synthetic peptides as antigens in order to identify target epitopes for MAbs raised against an intact, native protein may present a number of problems. This may relate to the probability that most native epitopes are discontinuous in native proteins (Barlow et al., 1986) and therefore they are not easily simulated using synthetic peptides.

Both anti-PHCS and MAb B showed higher binding to intact PHCS than to PCS-1. That may be expected in the case of anti-PHCS, since anti-PHCS serum contains antibodies to a number of epitopes of PHCS. The result obtained with PCS-1 suggests that there could be a range of conformations or an altered, conformation in free peptide.

Soluble PHCS inhibits anti-PHCS binding in a competitive sandwich ELISA when PHCS was the target antigen; in this case the PHCS is thought to exist in its native conformation when immobilized via the polyclonal antibody used for plate coating.

This suggests that the majority of the antibodies in anti-PHCS serum are against the native conformation of the enzyme PHCS. On the other hand, it has been found that soluble PHCS inhibited MAb B by 16% in competitive direct ELISA when the plate was coated directly by PHCS as the target antigen. This result indicates that MAb B is directed mostly against denatured PHCS, so that binding was not inhibited by native, soluble PHCS. These results are consistent with the findings of Brennand (1987), who concluded that MAb B was probably directed against denatured PHCS from the results of a number of assays using both direct and sandwich ELISA techniques, when the target was either partially denatured or in native form.

PCS-1 showed little inhibitory effect on MAb B binding, while no effect was detected with PCS-2 in competitive direct ELISAs. This suggests, that these peptides may not represent the site of interaction of this monoclonal antibody with PHCS. This observation supports the suggestion by Berzofsky et al. (1982) that MAbs against native protein were specific for structural features present only on the native protein but not on any of the CNBr fragments.

7.3 CROSS-REACTIVITY OF POLYCLONAL ANTIBODIES TO SYNTHETIC PEPTIDES

Peptides PCS-1 and PCS-2 were synthesised in this study and antibodies against them were produced. Anti-peptide sera showed a strict specificity for the immunizing peptide (with or without extension by G C residues), and did not react with alternative peptide (with or without G C residues) in direct ELISA assays using peptide-coated plates. Both anti-peptide sera reacted with PHCS, suggesting that the anti-peptide antibodies produced in this study bind to PHCS preferentially at the sites represented by their respective homologous peptides. Similar conclusions were reached by Burt et al. (1986) who prepared anti-peptide sera against synthetic peptides derived

from the sequence of rat immunoglobuline E.

Anti-PCS-1 serum was inhibited by PCS1-GC, the immunogen used in its production, but was only partially inhibited by PCS-1. This suggests that the majority of antibodies are directed against the PCS1-GC determinant. In the case of anti-PCS-2, it was found that both PCS-2 and PCS2-GC could inhibit the anti-serum to the same extent. This suggests that the majority of antibodies in anti-PCS-2 serum are directed against PCS-2, and do not involve the G C residues.

Having established the specifity of anti-peptide sera against synthetic peptides, their reactivity with fragments of PHCS was investigated. In immunoblots using fragments of PHCS produced by digestion with CNBr, anti-PCS-1 binds to a fragment of mol. wt. 10.5K, and in addition to larger fragments. The expected position of the PCS-1 determinant corresponds to a 9.6K fragment suggesting that the antibody may indeed bind to a region including 288-302 in fragments of native enzyme. Anti-PCS-2 was observed to bind to a fragment of 8.3K and also to larger fragments. A 6K fragment was expected to contain the PCS-2 determinant, so it is likely that anti-PCS-2 binds to residues 76-90 in fragments of native enzyme. In principle, following complete digestion of PHCS by CNBr each anti-peptide serum should bind to a single fragment. In these experiments, binding to higher mol. wt. fragments is probably due to incomplete cleavage of PHCS by CNBr. This suggestion could be confirmed by producing CNBr fragments of PHCS under conditions where complete cleavage is obtained. It is also possible that non-specific binding of serum immunoglobulins is responsible for some of the observed reactivity. Immunoblots using control sera (normal rabbit serum and rabbit anti-KLH) should indicate which bands, if any, are the result of non-specific binding.

Anti-PCS-1 was slightly blocked by soluble PHCS (in contrast to anti-PCS-2 which was blocked by up to 50%) in competitive, direct ELISAs with PHCS as the target antigen. The competitor, soluble PHCS, is in its native conformation while the target in this "direct" assay may be partially denatured enzyme. This suggests that most anti-

peptide antibodies in anti-PCS-1 react better with denatured PHCS, and therefore were not blocked by PHCS in its native form. Anti-PCS-2 may show more reactivity with the native enzyme, as it was blocked by PHCS to a greater extent. This suggests that some anti-peptide sera may react better with native determinants, while others react better with denatured determinants.

When studying the enzyme activity of PHCS, both anti-peptide sera showed no effect on activity although the enzyme activity was inhibited by anti-PHCS polyclonal antibodies. Since we know from the results of competitive ELISAs that anti-PCS-2 binds to the native enzyme, this suggests that binding of this antibody has no effect on the enzyme activity, while anti-PCS-1 was shown not to bind well to native enzyme. When staphylococcus aureus protein A was used to precipitate all enzyme-antibody complexes, an increased effect of anti-PCS-1 and anti-PCS-2 was seen. Under these conditions, anti-PHCS caused complete loss of activity from the solution.

Cross-reactivity of anti-PCS-1 and anti-PCS-2 with CS from different species (pig, E. coli and A. anitratum showed that the only binding was to CS of pig heart. Comparison of the alignments of CS sequences from these species suggests that this is the expected result, since there are major differences in amino acid sequence between these enzymes in the regions corresponding to peptides PCS-1 and PCS-2 (Fig. 6.13). On the other hand, this comparison suggests that yeast CS, and perhaps other eukaryotic CS, may bind these anti-peptide antibodies, due to the homology of these peptides with yeast CS as shown in Fig. 7.2. Pullen et al. (1985) showed that rabbit anti-serum to pig CS was cross-reactive with the enzyme from pigeon and, to a less extent, with that from Bacillus megaterium and E. coli. This indicates that there are other antigenic regions of the CS molecule which are more closely related between species than PCS-1 or PCS-2 peptides.

Fig. 7.2 Comparison of amino acid sequences of PHCS and yeast CS in the regions of the synthetic peptides

PCS-1

	288														302
PHCS	L	Q	K	E	V	G	K	D	V	S	D	E	K	L	R
Yeast CS	L	R	E	E	V	K	G	D	Y	S	K	E	T	I	E

PCS-2

	76														90
PHCS	K	M	L	P	K	A	K	G	G	E	E	P	L	P	E
Yeast CS	R	E	L	P	K	A	E	G	S	T	E	P	L	P	E

7.4 CONCLUSIONS

Synthetic peptides are extensively used in the field of immunology, hormone-receptor interactions and vaccine research. The general approach is to synthesize peptide homologs or analogs of segments of a larger polypetide or protein and evaluate their immunochemical characteristics. Because there have been limitations on the number of peptides which could be readily synthesized and tested, the choice of peptides has often been made on the basis of predictive algorithms which have appeared in the scientific literature in recent years (e.g. Thornton et al., 1986; Westhof et al., 1984; Hopp and Woods, 1981). When anti-sera are produced against synthetic peptides, a major objective is to obtain anti-sera which will react with the corresponding protein in its native state.

As pointed out earlier, methods used to predict protein epitopes can be divided into two main categories; those depending on the amino acid sequence and those depending on the conformational structure of the protein. So, when the amino acid sequence of a protein is available, it is sometimes possible to predict its epitopes by using the parameters based on the sequence.

It can be seen in the literature how the antigenic regions of simple proteins have been identified when their amino acid sequences are known (e.g. swMb; lysozyme, myohaemrythrin and TMVP). But the prediction of these epitopes will be more accurate if the conformational structure of the protein is available and the predicted regions agree with those identified from the amino acid sequence.

Although the prediction of the three-dimensional structure of a protein from its amino acid sequence remains one of the fundamental unsolved problems in molecular biology (Thornton, 1988), there is much information available for the structure and function of the model protein (PHCS) used in this study. Therefore, it has been possible to use both groups of predictive methods to identify which regions of PHCS are important in antigenic activity. In the case of PHCS, epitope prediction methods

based on sequence data suggested peptide antigens which were shown to produce anti sera capable of reacting with the native protein. Conformation-based methods generally confirmed these predictions.

Consequently, it can be concluded that these predictive approaches are potentially useful for the many protein sequences available, many of which are obtained from the gene sequences. Furthermore, conformational data of proteins, which are much more difficult to obtain and hence not usually available, may not be essential for epitope prediction. On occasions, the protein itself may not have been purified and characterised, making such data impossible to obtain.

Antisera raised against synthetic peptides (selected on the basis of various predictive methods) showed specificity for the immunizing peptide and cross-reactivity with the corresponding determinant in the intact protein. On the other hand, this work has produced no clear evidence that antisera or monoclonal antibodies against the intact enzyme are directed against those continuous determinants which are identified by epitope prediction methods. It is likely that anti-protein antibodies are directed against discontinuous epitopes which cannot be predicted by the methods used nor simulated by synthetic peptides corresponding to short segments of the linear amino acid sequence.

The work reported in this study suggests that the use of epitope prediction methods to identify potential synthetic peptide immunogens can be extended to the case of oligomeric proteins.

7.5 SUGGESTIONS FOR FURTHER WORK

1. It would be interesting to select and synthesise other peptides which are from a region not thought to be antigenic in the native CS molecule, and to produce anti-sera against these peptides. Such peptides could be selected from the interface region

between the two subunits of the dimer PHCS, or from regions which are buried within the monomeric subunit in its native conformation. The reactivity of these anti-peptide sera could be examined with native protein, protein fragments and other peptides to see whether they also will react with the native or denatured protein.

The results of these experiments would provide an additional test of the validity of the predictive methods and to investigate the relevance of this approach in the study of oligomeric proteins.

2. The research group is interested in a number of problems related to CS from a range of eukaryotic and prokaryotic species. One project is attempting to construct a 3D-structure for the hexameric CS from E. coli by computer homology modelling from its amino acid sequence with respect to the sequence and crystal structure of the dimeric PHCS. A dimer of the E. coli CS has been modelled so far and the assembly of three dimers into a hexamer is now being considered. It would be interesting to select peptides from the surface of this modelled dimer and to raise antibodies to them; cross-reactivity of these anti-peptide sera with the native, hexameric E. coli CS may then give clues as to which parts of the dimer are still exposed after association into the hexamers and which parts are buried.

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